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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF ARABINOGALACTAN PROTEIN 31 IN *ARABIDOPSIS*

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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF ARABINOGALACTAN PROTEIN 31 IN *ARABIDOPSIS*

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Arabinogalactan proteins (AGPs) are highly glycosylated cell wall proteins specific to plants. AGPs have been implicated in almost all aspects of plant development and defense responses, nevertheless, most of such studies are correlative. To define the specific functions of individual AGPs, direct evidence from analyses of genetic knock-out mutants of individual AGPs is required. Up to now, only a few AGPs have been demonstrated to have defined functions by mutant analyses. This dissertation identified a non-classical AGP (AGP31), described its expression and characterized the null mutant of *AGP31* in *Arabidopsis*. In *agp31* mutant, microarray analyses revealed that the expressions of genes encoding a subset of seed storage proteins (SSP): *CRU3*, *CRA1* and *OLEOSIN2* were induced. Further analysis showed that induction by *agp31* knockout was specific to these three SSP genes, indicating a novel pathway to regulate the SSP gene expression. Comprehensive characterizations of AGP31 were carried out. Yariv reagent staining and monosaccharide analysis of purified AGP31 showed that AGP31 was a *bona fide* galactose-rich AGP. The cell wall localization of AGP31 was confirmed by

expression of an AGP31::eGFP fusion protein. *AGP31* promoter-GUS reporter gene analysis showed that AGP31 was expressed in the vascular bundle throughout the plant, except in the flower. In the flower, it was expressed throughout the pistil except in the stigma. Detailed analysis showed that GUS staining occurred in all cell types in the vascular bundle of roots, while GUS staining was restricted to phloem cells in the inflorescence stem. *AGP31* mRNA was down-regulated by several stress treatments, including wounding, methyl jasmonic acid (MeJA) and abscisic acid (ABA). In response to MeJA treatment of whole seedlings, *AGP31* mRNA level decreased to about 30% of its original level within 8 hr and almost returned to its original level after 24 hr. Nuclei run-on assay showed that the down-regulation of *AGP31* mRNA upon MeJA treatment was due to reduced transcription. The strong preferential expression in vascular tissues and negative regulations by MeJA and ABA suggest that AGP31 may be involved in vascular tissue function both during development and the defense response.

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Chapter 1 Introduction

The plant cell wall is mainly composed of polysaccharides including cellulose, hemicellulose embedding in the pectin matrix, some structural proteins and enzymes. Modern biology views the cell wall as a dynamic structure instead of a static rigid box. During plant cell development and defense responses, plants vigorously adjust the cell wall structure to meet the various development and defense requirements. Cell wall proteins are believed to be involved in these crucial processes.

Among the cell wall proteins, cell wall structural proteins which include hydroxylproline-rich glycoproteins (HRGPs) and glycine rich proteins (GRPs) have long been treated as structural proteins as implied by their names. A number of studies have demonstrated that these proteins are involved in almost all aspects of plant physiology, from cell extension, pollen growth, and programmed cell death to plant pathogen defense responses.

HRGPs refer to the superfamily of proteins rich in proline/hydroxylproline and serine/threonines which usually form the highly repeated protein backbone. The hydroxylprolines are glycosylated by O-linked sugars, mostly being arabinofuranosyl (Ara*f*) and galactopyranosyl (Gal*p*) (Johnson et al., 2003). Based on the amino acid repeat unit of the protein backbone and glycosylation degree, HRGPs can be classified as

moderately glycosylated extensins, highly glycosylated arabinogalactan proteins (AGPs) and least glycosylated proline-rich proteins (PRPs). However, it is noteworthy that HRGPs are actually the continuum both in glycosylation pattern and amino acid repeat units. Some HRGPs have more than one type of amino acid repeat and should be treated as chimeric HRGPs (Hall and Cannon, 2002; Schultz et al., 2002).

Many studies of these proteins under normal growth condition or challenging with pathogens or abiotic stresses have revealed the specific expression patterns of various HRGPs (Swords and Staehelin, 1993; Guo et al., 1994; Kawalleck et al., 1995; Bonilla et al., 1997; Garcia-Muniz et al., 1998; Vignols et al., 1999). While suggesting HRGPs are involved in almost all aspects of plant physiology, these studies are all correlative. In order to assign a specific function to an individual HRGP, functional analysis of genetic knockout of an individual HRGP gene is required. Arabidopsis, due to its short life cycle and completely sequenced genome, is an excellent system to dissect the functions of HRGPs. Significant progress has been made by this approach. A few HRGPs have been demonstrated to have defined functions by genetic mutants (Baumberger et al., 2001; Hall and Cannon, 2002; Baumberger et al., 2003; Baumberger et al., 2003; van Hengel and Roberts, 2003; Acosta-Garcia and Vielle-Calzada, 2004; Gaspar et al., 2004; van Hengel et al., 2004; Yang et al., 2007). This dissertation was set to characterize and analyze the function of one HRGP—AtAGP31 (AGP31 hereafter) in Arabidopsis, which originally was annotated as PRP due to its similarity with another PRP in bean (PvPRP1). A brief introduction of every HRGP category is given below with emphasis on AGP.

Extensins

Among HRGPs, extensins are certainly the best characterized class. Extensins are defined by their unique amino acid repeat unit SP_{2-5}. Extensins are the moderately glycosylated HRGP. Carbohydrates comprise about 50% of the molecular mass (Johnson et al., 2003). It is believed that extensins are insolubilized in the cell wall after cessation of cell extension growth. Extensins can form inter- or intra- molecular cross-linking presumably through di-isodityrosine or isodityrosine bonds, respectively (Fong et al., 1992; Zhou et al., 1992; Brady et al., 1996; Brady and Fry, 1997). A recent study using a model extensin peptide system expressed in tobacco cells has identified the formation of di-isodityrosine intermolecular cross-links *in vitro* (Held et al., 2004). The cross-linking of extensins in cell walls is believed to provide the extra strength support to cell walls. Besides some extensin genes are induced during the plant defense response (including mechanical wounding and pathogen infections) and bacterial symbiosis (Showalter et al., 1991; Parmentier et al., 1995; Davies et al., 1997; Merkouropoulos et al., 1999; Merkouropoulos and Shirsat, 2003; Den Herder et al., 2007). Like all other HRGPs, extensins have been implied function in various plant growth and development processes, up to now only RSH (Root-Shoot-Hypocotyl defective) (Hall and Cannon, 2002) and LRXs (Leu-rich Repeat/Extensins) (Baumberger et al., 2001; Diet et al., 2006) have been demonstrated to have defined functions by analysis of genetic mutations and transgenic over-expressions.

RSH

The RSH gene encodes an extensin with 28 SP_4 repeat. A transposon insertion at the promoter region disrupts the expression of this extensin. The homozygous mutant *rsh/rsh* is defective in cytokinesis and cannot survive more than 3 week (Hall and Cannon, 2002). Due to its highly repeat nature in amino acids, the gene encoding RSH has been assigned to two extesins (EXT1 and EXTt4) (Hall and Cannon, 2002; Johnson et al., 2003). Over-expression of EXT1 does not cause prominent phenotypes in plant morphology except the stems were thicker than in wild type plants (Roberts and Shirsat, 2006). However, over-expression of EXT1 confers plants more resistant to the bacteria PstDC3000 infection (Wei and Shirsat, 2006).

LRX

LRX is a chimeric extensin family with Leu-rich repeats (LRRs) at the N-termini and extensin domains at the C-termini. In Arabidopsis and rice, there are 11 and 8 LRX family members, respectively (Baumberger et al., 2003). Besides, there are LRX members in tomato, maize and tobacco. Only mutants *atlrx1* and *atlrx2* have been shown to have phenotypes. AtLRX1 is involved in root hair morphogenesis. The null mutant *lrx1* caused frequent aborted, swollen and branched root hairs (Baumberger et al., 2001). AtLRX2 functions synergistically with AtLRX1. Although single mutant *atlrx2* does not have distinguishable phenotypes with the wild type, double mutant *atlrx1/atlrx2* shows more severe root hair abnormal phenotype than *atlrx1* single mutant. In addition, the phenotype of *atlrx1* can be complemented by *AtLRX1* promoter::*AtLRX2* and *AtLRX2*

promoter::*AtLRX1* constructs (Baumberger et al., 2003). The phenotype of *lrx1* is suppressed by mutations in the *RHM1* gene encoding a UDP-L-rhamnose synthase indicating that the mutation in *lrx1* can be repressed by modifications in pectin polysaccharides or other cell wall components (Diet et al., 2006).

PRPs

Proline-rich proteins (PRPs) refer to the HRGPs containing repeat unit PPVXK or its variants. Usually they are slightly or not glycosylated. The functions of individual PRPs have not been demonstrated (Johnson et al., 2003). However, like other HRGPs, PRPs have been correlated with various plant processes, suggesting they may function in these processes. In French bean, Nodulin 2 (ENOD2), a chimeric PRP with an extensin module, is localized in nodule parenchyma cells. It has been proposed that the ENOD2 may contribute to the modification of cell walls in these parenchyma cells, which form a barrier to the diffusion of oxygen (van de Wiel et al., 1990). In Arabidopsis, AtPRP3 is expressed in trichoblast cells at root-shoot junction and root differentiation zone. The expression of AtPRP3 can be induced or reduced by root hair promoting or suppressing treatment, respectively (Fowler et al., 1999; Bernhardt and Tierney, 2000). Mutants such as *ttg* and *gl2* causing ecotopic root hair development also enhance the expression of AtPRP3. These results suggest that the AtPRP3 contributes to the cell wall structure of root hairs (Bernhardt and Tierney, 2000).

AGPs

Arabinogalactan proteins (AGPs) are highly glycosylated cell wall structural proteins specific to plants. Carbohydrate moiety usually accounts for about 90% of AGP mass and occurs predominantly as type II arabinogalactans (Johnson et al., 2003). Operationally AGPs are defined by its ability to specifically bind a class of synthetic phenylazo dyes, the *β*-glycosyl Yariv reagents, such as *β*-glucosyl Yariv. Yariv reagent specifically binds to the carbohydrate moieties of AGPs, but the binding mechanism is unknown.

Based on amino acid sequence AGPs are classified into four classes: classical AGPs (incuding those with lysine-rich motifs), AG peptides, fasciclin AGPs and non-classical AGPs. Except for non-classical AGPs, all other AGPs are anchored to cell membrane through glycosylphasphatidylinositol (GPI) glycol-lipid anchors. The protein backbone of classical AGPs has three domains: an N-terminal secretion signal peptide which directs the protein outside the cell, a highly arabinogalactansylated proline/hydroxyproline rich domain and a C-terminal hydrophobic domain which directs the addition of a GPI anchor for attachment to the plasma membrane. AG peptides are small peptides about 12 aa long with arabinogalactan attached to it. Fasciclin AGPs, in addition to their proline rich motifs, contain fasciclin motifs which are involved in cell adhesions in animal cells. In the mature protein, both the signal peptide and the GPI anchor signal peptide are cleaved. The non-classical AGPs are essentially one kind of chimeric AGPs. They have both a proline-rich domain and a non-proline-rich domain.

None of the non-classical AGPs have been found to have a GPI anchor. The non-proline-rich motif can be Asn-rich or Cys-rich.

Using the Yariv reagent to disturb the functions of AGPs and analyzing the mutants of the individual AGP genes, AGPs have been shown to be involved in various plant developmental processes. These include cell-cell interactions, programmed cell death, somatic embryogenesis, pollen tube growth, xylem differentiation, female embryogenesis, fertilization, floral organ abscission, and apical cell extension (Showalter, 2001).

Besides their important role in plant development, AGPs have also been implicated in plant defense/stress response and hormone signaling (Li and Showalter, 1996; Suzuki et al., 2002; Park et al., 2003; Lamport et al., 2006). Using Yariv reagent to interfere with the functions of AGsP clearly showed that AGPs are involved in defense and hormone responses. In Arabidopsis cell culture, Yariv reagent can trigger wounding like response and program cell death (Gao and Showalter, 1999; Guan and Nothnagel, 2004). In barley aleurone protoplasts, Yariv reagent completely blocked the induction of alpha-amylase upon gibberellic acid (GA) treatment (Suzuki et al., 2002). Genetic manipulations of individual AGP genes also support the functions of AGPs in defense and hormone responses. A mutant of *AtAGP17* (*rat1*), encoding a lysine-rich classical AGP, is resistant to Agrobacterium transformation (Gaspar et al., 2004). The null mutant of *AtAGP30*, encoding a non-classical AGP, is more resistant to the inhibition of abscisic acid (ABA) during seed germination (van Hengel and Roberts, 2003). The mutant of

SOS5, encoding a chimeric protein of AGP and fasciclin (putative cell adhesion domains), shows hyper-sensitivity to salt stress (Shi et al., 2003). Lastly, many of AGP genes were shown to be regulated by defense and hormone treatments. In tobacco, the expression of *NaAGP4* is suppressed by wounding and fungal infection (Gilson et al., 2001). In tomato, *LeAGP1* is down-regulated by auxin and ABA while up-regulated by cytokinin (Sun et al., 2004). In French bean, the mRNA of *PvPRP1* (possibly an AGP, under investigation) is down-regulated upon fungal elicitor treatment and wounding (Sheng et al., 1991). *CsAGP1* mRNA was up-regulated in cucumber hypocotyls upon GA and auxin treatment and over-expression of CsAGP1 in tobacco promoted the shoot elongation, a typical response of GA treatment (Park et al., 2003). Salt stress up-regulated the periplasmic AGPs in *Acacia*, tomato, tobacco, and Arabidopsis cell cultures (Lamport et al., 2006).

AGPs, due to their high repeat nature in the proline-rich domains, are difficult to find by traditional blast program search, therefore it is difficult to identify the all the AGP family members. Using the biased amino acid composition method, which is based on calculation of the composition of predominant amino acids---- PAST (proline, alanine, serine and threonine) of AGPs, Schultz et al (2000) identified 26 classical AGPs and AG peptides and 21 fasciclin proteins in the Arabidopsis genome (Schultz et al., 2000; Johnson et al., 2003). While the classical AGPs can be identified by bioinformatics, the non-classical AGPs are very difficult to be identified by bioinformatics methods because of their highly diversified non-proline rich domains which usually reduce the calculated

PAST composition values. Up to now, only a few of them have been identified and characterized.

In this dissertation, I identified a non-classical AGP (AGP31) in Arabidopsis with cysteine-rich domain at C-terminus and histidine-rich domain at N-terminus. *AGP31* is a single gene in the Arabidopsis genome. Extensive characterization and phenotype analysis were carried out to analyze the function of AGP31. The mutant *agp31* does not have a noticeable phenotype when growing under normal condition. Expression pattern analysis correlated *AGP31* with the ABA and jasmonic acid (JA) signaling pathway. Microrrary analysis revealed that knockout of *AGP31* induced the expressions of genes encoding seed storage proteins CRU3, CRA1 and OLEOSIN2 in seedlings. To my knowledge, this is the first study showing that an AGP regulates the expression of seed storage proteins. The significance of this discovery has been discussed in this dissertation.

The chapter 2 of this dissertation is the isolation and characterizations of AGP31 protein from plants and comprehensive characterizations of *AGP31* gene expression patterns under normal condition and various stress treatments. The chapter 3 of this dissertation is the functional analysis of *AGP31* by analyzing the null mutant agp31. The chapter 4 of this dissertation is the prospective research directions of AGPs inspired by the studies of this dissertation.

Chapter 2 Characterization of *AGP31*[1]

Introduction

Plants respond to insect attack, mechanical damage and pathogen infection by regulating specific and overlapping sets of genes to provide wound-healing and protective functions. These responses widely involve both gene activation and repression. Both of these processes appear to be important in coordinating the antagonistic and synergistic interplay of different signaling pathways and downstream effectors to address the biotic stress (Lorenzo et al., 2003). For example, defense-induced salicylic acid promotes expression of various defense genes such as PR proteins while repressing the expression of genes under control of the jasmonate signaling pathway (Li et al., 2004). Recently, a bacterial elicitor has been shown to mediate repression of auxin signaling and enhance disease resistance through induced miRNA accumulation in Arabidopsis (Navarro et al., 2006).

Positive and negative regulation of gene expression, along with diverse nongenomic processes, contribute to the dynamic remodeling of the plant cell wall during responses to herbivory, wounding and infection. Changes in the wall often play a central role in the defense outcome (Schulze-Lefert, 2004). Callose formation at infection sites

[1] This chapter is the manuscript submitted to Plant Physiology titled as: A non-classical arabinogalactan-protein highly expressed in vascular tissues, AGP31, is transcriptionally repressed by methyl jasmonic acid in *Arabidopsis*

increased susceptibility to several fungal pathogens in Arabidopsis (Nishimura et al., 2003). Tomato plants treated by wounding, methyl jasmonate (MeJA) or systemin application released peptides from a cell wall hydroxyproline-rich glycoprotein that stimulated two well-known defense responses, extracellular alkalinization and accumulation of proteinase inhibitors (Nishimura et al., 2003; Narvaez-Vasquez et al., 2005). Among roles identified for cell wall proteins, the rapid oxidative cross-linking of selected cell wall proteins to strengthen the cell wall against pathogen attack has been widely documented in many plants. Hydroxy/proline-rich proteins (HRGPs) such as extensins and proline-rich proteins (PRPs) have been identified as undergoing cross-linking, particularly those rich in tyrosine (Bradley et al., 1992; Brown et al., 1998). Tyrosine residues in these proteins have been long postulated to form intermolecular linkages responsible for insolubilization of the proteins in the wall during the defense response. A recent study using a model extensin peptide system expressed in tobacco cells has identified the formation of di-isodityrosine intermolecular cross-links *in vitro* (Held et al., 2004). Besides oxidative cross-linking of extensins and proline-rich proteins, oxidative cross-linking was also demonstrated by formation of high molecular weight complexes of plasma membrane associated arabinogalactan-proteins (AGPs) after wounding in sugar beet leaves (Kjellbom et al., 1997). It has been hypothesized that other HRGPs and PRPs with lower tyrosine contents do not cross-link and may not contribute to the defense response (Sheng et al., 1991). The cucumber PRP1 remained soluble after wounding, elicitor and hydrogen peroxide treatments and was instead

suggested to participate in the deposition of protective silica at infection sites (Kauss et al., 2003).

In a number of cases, changes in HRGP and PRP gene expression have been found to correlate with the presumed function or lack of function in protection during the plant defense response. Accordingly, specific HRGP and PRP mRNAs whose encoded proteins perform postulated or demonstrated adaptive roles are induced by defense-related stimuli (Bradley et al., 1992; Kauss et al., 2003; Pearce and Ryan, 2003). In contrast, several mRNAs encoding low tyrosine HRGPs and PRPs are down-regulated (Sauer et al., 1990; Sheng et al., 1991). For the latter group, it has been postulated that the genes are down-regulated to reduce synthesis of unneeded proteins to conserve energy and perhaps enhance the cell wall structure (Sheng et al., 1991; Mehdy, 1994). While down-regulation of numerous genes during plant defense responses has been observed, particularly with the advent of microarray technology, the molecular mechanisms generating the down-regulation are poorly understood. The mechanism of down-regulation of a low tyrosine PRP mRNA has been examined for the French bean (*Phaseolus vulgaris*) gene, *PvPRP1*. The *PvPRP1* mRNA was destabilized in elicitor treated cells such that the mRNA half-life decreased from about 60 hr in unelicited cells to approximately 45 min in elicited cells. In contrast, there was no change in the rate of *PvPRP1* transcription (Zhang et al., 1993). While progress has been made in identifying a RNA binding protein which binds to the *PvPRP1* mRNA *in vitro* (Zhang and Mehdy, 1994), the lack of facile genetic approaches, a sequenced genome and many resources such as mutant insertion lines in the French bean system hamper elucidation of the

regulatory pathway. Identification of a PRP gene in Arabidopsis homologous to the bean *PvPRP1* gene may provide a good candidate to examine defense-regulated gene repression in the model Arabidopsis system.

In this study, a low tyrosine PRP was identified in Arabidopsis whose mRNA was down-regulated by various defense-related stimuli: wounding, methyl jasmonate (MeJA) and abscisic acid (ABA) treatments. MeJA repression of the mRNA level was COI1 dependent and occurred at the level of transcription. This PRP is better classified as a non-classical AGP, on the basis of hallmark reactivity with β-glucosyl Yariv reagent and having an extensive carbohydrate moiety rich in galactose and ararbinose. Hence, the gene was named *AGP31*. Analysis of *AGP31* promoter-*GUS* reporter gene expression in transgenic plants revealed a striking association of expression with the vascular tissue throughout the plant except for more widespread expression in flowers. The strong preferential expression in vascular tissues suggests that AGP31 may be involved in vascular tissue function both during the defense response and development.

Results

AGP31 encodes a chimeric non-classical arabinogalactan-protein

A BLAST P search was conducted to identify Arabidopsis proteins with features and protein organization similar to the French bean PvPRP1 protein, including low tyrosine content (generally less than 5%). The gene with the greatest similarity throughout the encoded protein length was At1g28290. Using RT-PCR, we obtained a cDNA clone spanning the complete open reading frame of At1g28290. DNA sequencing

confirmed the annotation of At1g28290 at http://www.arabidopsis.org. At1g28290 has one intron and a 208 nt 3' UTR deduced from the cDNA sequence. At1g28290 encodes a 359 amino acid proline-rich protein with four distinct domains: a putative signal peptide followed by a histidine-rich domain, middle repetitive proline rich domain and a C-terminal non-proline rich domain (Fig. 1.1A). In the proline-rich domain, there are only five types of amino acids, with 45.9% proline, 18.2% lysine, 15.7% valine, 6.3% alanine and 3.8% tyrosine. These amino acids form different exact repeat units. There are two long repeats with 31 amino acids overlapping with four shorter 20 amino acid repeats (Fig. 1.1A). These repeats are essentially composed of different variants of the basic repeat unit PP(A/V/T)(K/Y). This degenerate quadri-peptide is different from the typical penta-peptide PPVX (K/T) of other PRPs, such as several AtPRPs in *Arabidopsis* (Fowler et al., 1999) and ENOD11 in *Medicago* (Journet et al., 2001), and is characteristic of a class of non-classical AGPs and AGP-like proteins. Also in common with these proteins, the At1g28290 protein has a C-terminal region with six well conserved cysteines that has been named PAC (present in PRP and AGP, containing Cysteine) (Baldwin et al., 2001). This domain also exists in the OLE E 1 pollen allergen in olive.

The protein sequence of At1g28290 suggested that it may encode a non-classical AGP. Preceding the His-rich domain, there is a module APAPAP and there are 6 single AP duplets scattered in the PRP domain (Fig. 1.1A). Increasing evidence suggests these AP modules are sites of carbohydrate attachment in AGPs (Tan et al., 2004). In addition to these putative *O*-glycosylation sites, there are two putative N-glycosylation sites in the

PAC region (Fig. 1.1A). Based on these analyses, we postulated this protein might be a non-classical AGP.

Further supporting evidence was provided using the PAC domain sequence to blast the Genebank database. This identified several putative homologs among different species. Among these putative homologs, we searched for proteins which have a similar structure: predicted signal sequence, N-terminal histidine-rich stretch, proline-rich region with the repeat PPXX, and PAC domain. After applying these criteria, we identified several proteins, PvPRP1 (French bean), HyPRP1 (cotton), DcAGP1 (carrot), NaPRP4, NtTTS1 and NtTTS2 (tobacco), which we consider to be homologs of At1g28290 (Fig. 1.1B). Among these homologs, DcAGP1, NaPRP4, NtTTS1 and NtTTS2 have been shown to be bona fide non-classical AGPs (Cheung et al., 1995; Sommer-Knudsen et al., 1996; Baldwin et al., 2001). In the Arabidopsis genome, there are two genes, AtAGP30 and At2g34700, showing similarity with At1g28290. AtAGP30 (van Hengel and Roberts, 2003) shows the highest similarity with At1g28290 but it has no histidine-rich domain. At2g34700 has only the PAC domain without histidine and proline-rich domains. Based on these analyses, we concluded that At1g28290 is the only gene in this non-classical AGP subclass in Arabidopsis genome.

To determine whether At1g28290 is a bona fide AGP, we used the 35S promoter to over-express the At1g28290 protein fused with a 6X myc tag at the C-terminal end in transgenic plants. A cell wall fraction was isolated from 7-day-old seedlings. Proteins released by high salt extraction of this fraction were incubated with ß glucosyl Yariv reagent, a compound that selectively precipitates AGPs. Western blot analysis of the

AGP fraction using an anti-myc antibody detected the fusion protein and an endogenous, nonspecific 50 kD protein (Fig. 1.1C). This result demonstrates that At1g28290 is a bona fide AGP. The myc fusion protein detected by the antibody ran by SDS-PAGE as a broad high MW band of about 170-200 kD whereas the predicted MW of the mature At1g28290 protein alone is 38 kD. In addition to this experiment, we isolated the myc fusion protein from the high salt cell wall protein fraction by Ni-NTA affinity purification, capitalizing on the fact that there is a histidine-rich stretch near the N-terminus of At1g28290. The resulting enriched protein fraction spotted on nitrocellulose stained positively with Yariv reagent (data not shown). Based on these analyses, we concluded that At1g28290 encodes a non-classical AGP and named it AGP31.

AGP31 is a galactose-rich AGP

To examine its carbohydrate composition and likely hydroxylation of prolines, we purified native AGP31 protein from transgenic plants over-expressing native AGP31. Transgenic lines were generated by introducing a genomic fragment which contains about 3 kb upstream of translation start codon and about 700 bp downstream of translation stop codon of AGP31 gene. Due to the nature of *Agrobacterium* transformation, multiple copies of fragments are frequently introduced into plants and can exhibit higher mRNA levels. Plants of a transgenic line that showed a higher mRNA level than wild-type grown in liquid MS media were used to isolate the high salt eluted cell wall protein. The AGP31 was purified by a Ni-NTA metal affinity column from high salt eluted cell wall protein and an aliquot subjected to trifluoromethanesulfonic acid

(TFMS) hydrolysis, a treatment that removes the majority of carbohydrate in glycoproteins. The Coomassie stained gel showed that acid hydrolysis resulted in a dramatic shift from the broad high MW band to a single prominent band of 45 kD in the deglycosylated sample (Fig. 1.2). It is noteworthy that the innermost asparagine-linked *N*-acetylglucosamine of *N*-linked oligosaccharide is resistant to TFMS treatment (Edge, 2003). Deglycosylated AGP31 ran at apparent MW 45 kD higher than the predicted MW 38 kD, indicating that the *N*-glycosylation indeed exists in AGP31. The identity of the band was identified by MALDI-TOF MS which covered 61% of peptides in AGP31. No other protein can be identified by the MALDI-TOF MS from the single band. Together, these findings demonstrate that AGP31 is a glycoprotein and that a highly purified preparation of AGP31 has been obtained.

Amino acid composition analysis of purified, deglycosylated AGP31 showed approximately equal amounts of proline and hydroxyproline (Table 1.1). Another non-classical AGP, NaPRP4, which is the homolog of AGP31 in *Nicotiana alata*, also has about equal amounts of proline and hydroxyproline (Sommer-Knudsen et al., 1996). Monosaccharide composition analysis showed that purified AGP31 contains mostly galactose (80.9%), with lesser amounts of arabinose (13.5%), xylose (2.1%), fucose (1.3%) and mannose (1.3%) (Table 1.2). In addition, very low amount of glucose (0.5%) and glucuronic acid (0.5%) were detected. Again, AGP31 is very similar to NaPRP4 which has a glycosyl composition of 83% galactose, 6.8% arabinose, 3.5% xylose and 1.5% mannose (Sommer-Knudsen et al., 1996). Besides AGP31's interaction with the

Yariv reagent, the glycosyl composition analysis provided an independent confirmation that AGP31 is an AGP.

AGP31 mRNA is repressed by wounding, MeJA and ABA

In order to investigate *AGP31* gene expression during the defense response, the leaves of 4-week-old plants were mechanically wounded and directly wounded leaves were harvested at various times. RNA blot analysis shows a decrease in *AGP31* mRNA level over the 8 hr time course (Fig. 1.3A). The 1.35 kb size of the *AGP31* transcript matches the expected size based on the cDNA sequence. The glutathione-S-transferase 1 (*GST1*) transcript was also hybridized as a known wounding-induced mRNA while the ubiquitin 10 (*UBQ10*) transcript was monitored to show equal loading. As jasmonic acid and its derivatives are well known secondary messengers induced by wounding, we also examined the effect of methyl jasmonate (MeJA). MeJA treatment resulted in a decrease in the *AGP31* mRNA to about 30% of the original level by 8 hr (Fig. 1.3B). After 12 hr, the *AGP31* mRNA level began to recover but remained below its original level through the 24 hr time course. In contrast, control plants treated with the solvent exhibited only a slight decrease in *AGP31* mRNA. The marked induction of lipoxygenase 2 (*Lox2*) mRNA, a known MeJA regulated mRNA, demonstrated effective MeJA exposure. Control plants treated with solvent showed a low level induction of *Lox2* mRNA, especially at early time points. However, compared with MeJA treated plants, the response was much lower and diminished after 4 hr.

ABA has been shown to be involved in some plant defense responses and is widely active in the response to water stress and other abiotic stresses. In order to determine the expression pattern of *AGP31* mRNA in response to ABA, we treated seedlings with ABA and harvested after 8 hr. Northern blot analysis showed that the *AGP31* mRNA was also repressed by ABA treatment (Fig. 1.3C).

The repression of AGP31 mRNA is primarily COI1 dependent

COI1 has been shown to play a pivotal role in jasmonate signaling. Based on microarray data, it has been estimated that about half of jasmonate signaling involves the action of this protein which encodes an F-box protein as part of the E3 ubiquitin ligase complex (Devoto et al., 2005). Although a slight repression was still observed, *AGP31* mRNA repression in response to MeJA was dramatically abolished in the *coi1-1* mutant compared to the response in wild type plants, indicating the *AGP31* mRNA repression is mainly dependent on the COI1 pathway (Fig. 1.4). Interestingly, the basal level of *AGP31* mRNA is similar in untreated plants of both genetic backgrounds.

The repression of AGP31 mRNA occurs primarily at the transcriptional level

In French bean, the repression of *PvPRP1* mRNA is due to mRNA stability regulation with no change in the rate of transcription (Zhang et al., 1993). To evaluate these two parameters for *AGP31* regulation, we performed a nuclear run-on assay to measure the transcription rate of the *AGP31* gene after MeJA treatment. Because Arabidopsis cultured cells can provide a large quantity of homogeneous cells, the run-on assay was carried out with cultured cell nuclei. Figure 1.5A shows that MeJA treatment

of cultured cells resulted in *AGP31* mRNA repression similar to the response in whole plants. Nuclei from the same batches of cells were used for run-on transcription assays. The results show that *AGP31* mRNA repression reflected a decreased transcription rate (Fig. 1.5B). Moreover, the magnitude of transcription reduction (to about 30% of the original level) correlates well with the observed reduction in the steady-state mRNA level to approximately 30% of the original level. A known MeJA induced gene, Ethylene Response Factor 1 (*ERF1*), showed increased transcription while the rates of Actin 2 (*ACT2*) and *UBQ10* transcription were essentially unchanged.

To investigate whether mRNA stability regulation may also play a role, the *AGP31* 3'-UTR was inserted downstream of a LUC^{+} reporter coding sequence. *Agrobacterium* mediated transformation generally results in the concatenated insertion of several T-DNA copies at a single locus. To avoid possible generation of double stranded RNAs and RNA interference processes that could variously impact the steady state mRNA levels, transgenic plants were screened to identify single copy insertions by Southern blot as described by Forsbach et al. (2003). Analysis of three lines of transgenic plants containing single copy insertions showed that the LUC^{+} reporter mRNA level remained approximately constant in MeJA treated plants whereas the endogenous *AGP31* and *Lox2* mRNAs showed the expected regulation (Fig. 1.6). The results suggest that the MeJA induced down-regulation of *AGP31* mRNA does not involve mRNA destabilization through the 3'-UTR sequence. While mRNA stability regulation commonly operates through the 3'- UTR (Bevilacqua et al., 2003), the present evidence does not rule out a role for another part of the transcript or the 3'-UTR plus another

sequence in conferring MeJA induced mRNA stability regulation. Together with the run-on data, our results support a predominant regulation by repression of transcription.

AGP31 is localized in the cell wall

AGP31 protein is predicted to be a cell wall or secreted protein based on its putative signal sequence and past findings of these locations for numerous AGPs. To establish the subcellular localization of AGP31, *eGFP* was fused to the C-terminal end of *AGP31* to form an AGP31::eGFP fusion construct under the control of the 35S promoter. The constructs were stably expressed in Arabidopsis transgenic plants and also bombarded into onion epidermal cells to express the fusion protein transiently. Figure 1.7A and B show confocal microscope views of the same root section from stable transgenic Arabidopsis plants. The eGFP signal indicated that the fusion protein was localized to the cell periphery (Fig. 1.7, A and B). In bombarded onion epidermal cells, the eGFP alone construct showed an intracellular localization, including a commonly observed presence in the nuclei (Fig. 1.7, C and D). In order to distinguish between cell wall and cell membrane locations, the transiently transformed cells were treated with 0.8 M mannitol to induce plasmolysis. As shown in Figure 1.7E, the mannitol treatment effectively contracted the plasma membrane from the cell periphery in a cell that expressed eGFP alone. The corresponding fluorescence image (Fig. 1.7F) showed the eGFP fluorescence was also contracted with the cytoplasm. In contrast, in a plasmolyzed transgenic cell expressing the AGP31-eGFP fusion protein, a significant portion of the fusion protein was found at the cell periphery (Fig. 1.7H), indicating cell wall localization.

Some fusion protein was also seen more faintly inside the cell and at the plasma membrane which would be consistent with intermediate locations of the protein during its biogenesis. The AGP31-myc fusion protein was also highly enriched in the high salt elution fraction from partially purified cell walls (data not shown). In work from other researchers, AGP31 was also identified in a cell wall proteomics study (Feiz et al., 2006) and this is consistent with our data indicating that AGP31 is a cell wall protein.

AGP31 is expressed primarily in the vascular bundle and in the flower

Toward understanding the biological function of this protein during defense and normal development, we characterized the *AGP31* mRNA expression level in roots, leaves, stems, and inflorescences of Arabidopsis plants. RNA blot analysis showed *AGP31* mRNA was expressed in all parts of the plant, with the roots and inflorescences having the highest expression levels (Fig. 1.8A). In order to determine the expression pattern of the *AGP31* gene more precisely, we made transgenic plants harboring the bacterial *uidA* gene (*GUS*) under the control of a 1.4 kb fragment extending upstream from the *AGP31* start codon. This construct is termed the *AGP31* promoter-*GUS* reporter construct (P_{AGP31}::*GUS*). The GUS expression pattern based on histochemical staining for GUS activity revealed that the *AGP31* promoter-*GUS* reporter gene was predominantly expressed in the vascular bundles throughout the plants (Fig. 1.8, B-F). In flowers, apart from staining of vascular bundles in sepals and stamen filaments, GUS was also expressed throughout pistils except for the stigmas (Fig. 1.8C). In roots, the GUS stain was predominant in the vascular bundles without appearance in the root tip regions (Fig.

1.8, D and E). The cross section of the root showed GUS activity was broadly distributed through the vascular bundle, including phloem and primary xylem (Fig. 1.8F).

Discussion

Wounding and MeJA have been shown to induce dozens of genes while repressing an approximately equal number of other genes (Devoto et al., 2005). However, the mechanisms of gene repression are poorly understood. The defense-related remodeling of the plant cell wall involves the activation of some cell wall protein genes while others are repressed, providing an opportunity to understand molecular regulatory mechanisms that have evolved in the context of altering cell wall properties for improved disease resistance. In this study, we have identified a non-classical AGP gene, *AGP31,* whose mRNA is down-regulated by MeJA, wounding and ABA. We further showed that MeJA suppression is primarily due to transcriptional repression and the signaling pathway involves a central JA regulator, COI1. In addition, *AGP31* expression during normal development showed a striking association with the vascular tissue and the pistil exclusive of the stigma.

As part of our interest in understanding gene repression during the defense response, we identified the At1g28290 gene in Arabidopsis as a close homolog to the known French bean elicitor and wounding down-regulated proline-rich protein gene, *PvPRP1* (Sheng et al., 1991; Zhang et al., 1993; Zhang and Mehdy, 1994). More broadly, AGP31 belongs to a subclass of non-classical AGPs discussed by Roberts and co-workers (Baldwin et al., 2001). This subclass includes a cotton PRP (HyPRP1), a carrot AGP

(DcAGP1), a French bean PRP (PvPRP1), two tobacco AGPs localized in the transmitting tissue of the style (NtTTS1, NtTTS2) and also the *Nicotiana alata* AGP, NaPRP4/GaRSGP. Although PvPRP1 (Sheng et al., 1991) and HyPRP1 were previously identified as PRPs in their database annotations, their features suggest that they may also be AGPs in this class. There are four common features of this family: a putative signal peptide, a His-rich domain near the N-terminus of the mature protein after presumed cleavage of the signal peptide, a proline-rich domain with the unique repeat PPXX and a C-terminal domain called PAC which has 6 well conserved cysteines (Baldwin et al., 2001). This PAC region has homology to the pollen allergen OLE E 1.

Although the functions of these domains remain to be clarified, there is some evidence for roles in diverse aspects of plant development and defense. The His-rich domain usually functions in metal binding in other proteins. For example, the His-rich domain of citrus dehydrin binds Cu^{2+} (Hara et al., 2005). In soybean, the His-rich domain is necessary for Ni^{2+} binding to Eu3, a Ni^{2+} binding GTPase that is required for urease activity (Freyermuth et al., 2000). Therefore, we postulate that the His-rich domain in AGP31, which we have shown can bind to Ni^{2+} *in vitro*, may possess a metal ion binding activity in the cell wall *in vivo*. Alternatively, the His-rich domain may interact with pectin as has been previously postulated (Baldwin et al., 2001). In rice, genes containing OLE E 1 domain also have other domains involved in various aspect of plant physiology such as auxin response, defense response and ion binding (Jiang et al., 2005). The tomato LeLAT52 protein has only the OLE E 1 domain, and has been shown to interact with a

leucine repeat receptor kinase and is essential for pollen hydration and pollen tube growth (Tang et al., 2002).

It is worth noting that although members of the non-classical AGP subclass discussed in this paper have similar overall structures, differences in their spatial expression and regulation suggest there may be different and common functions. For example, carrot *DcAGP1* mRNA is unaffected by wounding (Baldwin et al., 2003) whereas *AGP31* and bean *PvPRP1* mRNAs are both down-regulated (Sheng et al., 1991; Baldwin et al., 2001). The tobacco *TTS1* and *TTS2* mRNAs are highly localized to the transmitting tract of the style have been implicated in pollen tube growth guidance (Cheung et al., 1995). On the other hand, *AGP31* is primarily expressed in vascular tissues throughout the plant and broadly in the pistil. As AGP31 appears to be the only member of this subclass of non-classical AGPs in Arabidopsis based on our database searches, analysis of its function during defense and development may be less complicated by gene redundancy.

The physiological functions of non-classical AGPs have remained more elusive than individual classical AGPs whose functions have begun to be uncovered by genetic analyses (Yang et al., 2007). This is partially due to difficulties in identifying non-classical AGPs due to their highly variable C-terminal regions (Schultz et al., 2002). Surprisingly, in Arabidopsis, only one other non-classical AGP has been previously identified, AtAGP30. In comparison to AGP31, AtAGP30 is missing the His-rich domain but possesses a similar repetitive proline-rich domain and PAC domain. This gene is expressed primarily in the atrichoblasts of root epidermal cells (van Hengel et al., 2004).

A transposon insertional mutant of AtAGP30 exhibited resistance to ABA inhibition of seed germination and abnormal somatic embryogenesis (van Hengel and Roberts, 2003). ABA is a phytohormone involved in the plant stress responses. Here, we showed AGP31 was repressed by ABA treatment suggesting it might function in stress responses. Interestingly, two chimeric AGPs, xylogen1 and xylogen 2, stimulate the organization of xylem elements in zinnia and Arabidopsis. Xylogen1 and xylogen 2 are apparently chimeras of non-specific lipid transferase protein (nsLTP) and AGP (Motose et al., 2004).

A common feature among all AGPs is that they are highly glycosylated cell wall or secreted proteins in which the carbohydrate moiety usually accounts for about 90% of AGP mass. The carbohydrate exists predominantly as type II arabinogalactans and shows distinctive binding to a class of synthetic phenylazo dyes. Binding one member of this class of dyes, β-glucosyl Yariv, is a diagnostic parameter used to classify proteins as AGPs. Our findings that AGP31 can be precipitated by Yariv reagent and that it is a glycoprotein with abundant galactose (80.9%) and substantial arabinose (13.5%) establish it as a bona fide AGP. Western blot analysis of the AGP31-myc tag protein from transgenic whole seedlings revealed a much higher MW protein (170-200 kD) than expected based on the deduced amino acid sequence (38 kD), consistent with substantial glycosylation, at least at the stage of development used in these experiments. Compared with classical AGPs, AGP31 and NaPRP4 have less hydroxyproline and galactose is the predominant monosaccharide residue. For example, in LeAGP1 (Zhao et al., 2002) and gum arabic glycoprotein (Goodrum et al., 2000), most prolines are hydroxylated and there are about equal amounts of arabinose and galactose.

Using the promoter-*GUS* fusion technique, we showed that the expression of *AGP31* was localized to vascular bundles throughout the plant, including phloem and primary xylem cells. *AGP31* mRNA repression by MeJA and ABA correlates spatially with the vascular tissue being a major site for biosynthesis of both phytohormones (Stenzel et al., 2003; Koiwai et al., 2004). During the defense response, we postulate that AGP31 may not participate in the oxidative cross-linking of cell wall proteins and hence its synthesis is curtailed. Oxidative cross-linking in the vascular system as part of the defense response has been documented in vascular parenchyma cells in tomato in response to wounding, systemin and MeJA (Orozco-Cardenas et al., 2001).

We conclude that down-regulation of *AGP31* mRNA by MeJA is achieved by the combination of repression of transcription and a relatively short mRNA half-life (less than 4 hr is suggested by the kinetics of mRNA loss). The mRNA half-life may be constitutively short or regulated from a longer half-life found in untreated cells. However, the finding that the *AGP31* 3'-UTR did not impart MeJA-induced instability to a LUC^{+} reporter transcript makes it more likely that mRNA stability regulation is not a major factor as a common region controlling mRNA stability is the 3'-UTR (Bevilacqua et al., 2003). The predominant transcriptional repression of *AGP31* contrasts with the mRNA stability regulation that down-regulates the homologous *PvPRP1* mRNA in French bean. The different regulation mechanisms may reflect the different functions of these non-classical AGPs in different plants.

The transcription factor(s) mediating *AGP31* transcriptional repression by MeJA is unknown. A number of Arabidopsis transcription factors have been identified that

repress several JA-inducible genes including *ERF4* (McGrath et al., 2005), *WRKY70* (Li et al., 2004), *JIN1/MYC2* (Lorenzo et al., 2004). The *WRKY70* transcript was repressed by JA with kinetics similar to the loss of the *AGP31* mRNA (Li et al., 2004). The observed repression of *AGP31* mRNA by ABA also may be due to transcriptional and /or post-transcriptional processes. An ABA regulated transcriptional repressor, AtERF7, has been characterized (Song et al., 2005). There is also precedent for post-transcriptional regulation by ABA (Borsani et al., 2005; Nishimura et al., 2005). ABA repressed another group of AGP mRNAs: fasciclin-like AGP mRNAs encoding FLA 1, 2, 8 in Arabidopsis (Johnson et al., 2003). Identifying the function of AGP31 in diverse tissues and during defense and identifying the signaling pathway leading to gene repression will be aided by the analysis of *AGP31* mutants and use of other Arabidopsis genetic resources.

Materials and Methods

Plant materials and growth condition

Arabidopsis were grown on agar plates with 0.5XMS salts, 1% sucrose, and 0.8% phytoagar (Bio World) under continuous light at 20-23 °C. The wild type plant is Col-0. *coi1-1* mutant was a gift from Dr. J.G. Turner (University of East Anglia, Norwich, UK). Arabidopsis cell culture was obtained from Dr. W. F. Thompson (North Carolina State University, Raleigh, USA).

Cell wall protein isolation

The cell wall fractions were isolated from 8 g 11-day seedlings according to Feiz et al (2006) with some modifications. Briefly, plants were ground into powder in liquid

nitrogen and extracted with 2 volumes of extraction buffer (5 mM Sodium Acetate pH 4.0, 0.4 M sucrose with protease inhibitor, 0.1 g PVPP/g tissue) at 4 °C for 30 min. The extracts were centrifuged at 1000 g for 15 min at 4 °C. After removing the supernatant, the pellets were sequentially washed with 5mM sodium acetate, pH 4.0 with 0.6 M sucrose and 1 M sucrose respectively. Finally, the pellets were washed with 5 mM sodium acetate (at least 5 times) and considered to be the cell wall fraction.

AGP isolation

The AGPs were isolated from high salt eluted cell wall proteins according to Schultz et al (2000) with some modifications. The high-salt eluted cell wall proteins were extracted by incubating the cell wall fraction with high salt extraction buffer (5 mM sodium acetate, pH 4.0, 1 M sodium chloride, protease inhibitor) at 4 °C for 30 min. Then the samples were centrifuged at 5000 g for 15 min at 4 °C, the supernatants were transferred to new tubes and dialyzed against water overnight at 4 °C. The dialyzed cell wall proteins were freeze-dried and dissolved in 1mL 1% sodium chloride. To isolate the AGP fraction from cell wall protein, Yariv reagent was added to the cell wall protein at 1mg/mL and incubated at 4 °C overnight. To collect the Yariv-AGP complex, the mixtures were centrifuged at 4 °C for 1 hr. The resulting pellets were washed 3 times with 1% sodium chloride and once with methanol. After air drying at room temperature, the pellets were dissolved in minimal DMSO. To release the AGP from Yariv reagent, water and sodium hydrosulfite were added until the solution became pale yellow. The solutions were then dialyzed against water overnight at 4 °C and freeze-dried. Finally,

the AGP fractions were dissolved in water and the concentrations were determined by gel diffusion assay using Gum Arabic as standard (van Holst and Clarke, 1985).

AGP31 isolation

In order to isolate native AGP31, transgenic plant over-expressing native AGP31 was generated by transforming plants with *Agrobacterium* carrying an EcoRI and HindIII genomic fragment from BAC F3H6 (obtained from The Arabidopsis Information Resource). This fragment contains 3130 bp upstream of *AGP31* translation start codon and 701 bp downstream of *AGP31* translation stop codon, respectively. A transgenic plant over-expressing the AGP31 was used to isolate the native AGP31. Plants were grown in MS liquid media supplemented with 1% sucrose and full strength of MS vitamins (Czako et al., 1993). 30 g plants were used to isolate cell wall protein as describe above. The high salt eluted cell wall protein was dialyzed against water over night. One-tenth lysis buffer (100 mM NaH_2PO_4, PH 8.0, 500 mM imidozone, 3 M NaCl) was added to cell wall protein fraction. The AGP31 was isolated from cell wall protein by Ni-NTA column (Ni-NTA Fast Start Kit, QIAGEN) following the manufacturer's instructions. Finally, the isolated AGP31 was dialyzed against water and concentrated by Amicon Ultra-15 (Millipore).

AGP31 deglycosylation

Isolated AGP31 was deglycosylated by trifluoromethanesulfonic acid (TFMS) method using the GlycoProfile™ IV Kit (Sigma). Deglycosylated AGP31 and native AGP31 were separated by SDS-PAGE on 15% Tris-glycine gel.

Protein identification

Protein bands were excised from Coomassie blue stained gel and digested by trypsin. MALDI-TOF MS was carried by the core facility service of The University of Texas at Austin to identify the protein bands.

Amino acid composition analysis

The amino acid analysis was carried out by AAA Service Laboratory, Inc. (Damascus, OR 97089) as described (Roach and Gehrke, 1970; Simpson et al., 1976).

Monosaccharide composition analysis

Monosaccharide composition analysis was carried out by the Complex Carbohydrate Research Center (University of Georgia, Athens, GA) as described (Merkle and Poppe, 1994).

Western blot

7.5 μg AGP from wild type and transgenic plants were loaded on 4-15% gradient gel and were blotted to nitrocellulose membrane (BioRad) by a semi-dry transfer unit (Bio-Rad). The membranes were blocked in 5% BSA in PBST (PBS buffer+0.5% Triton 100). The primary antibody, anti-myc polyclonal antibody (Sigma), was diluted 1:1000, and the secondary antibody was diluted 1:25000. The signal was captured using the enhanced chemiluminescent method following the manufacturer's instruction (Pierce).

Wounding, MeJA and ABA treatments

4-week-old plants grown in soil were used for wounding treatment. About 1/3 of the leaves were pressed with a pliers and only the wounded leaves were used for RNA extraction. For MeJA treatment, 11-day-old plants grown vertically in standard agar

plates (0.5XMS salts, 1% sucrose, and 0.8% phyto agar) were sprayed with 500 μM MeJA or transferred to 50 μM MeJA plates (0.5X MS salts, 1% sucrose, and 0.8% phyto agar, 50 μM MeJA). For ABA treatment, 11-day-old plants were transferred to 10 μM ABA plates (0.5XMS salts, 1% sucrose, and 0.8% phyto agar, 10 μM ABA). After treatment for the indicated times, whole plants were harvested and frozen in liquid nitrogen.

RNA isolation and Northern blot

Total RNA was isolated from plant and cell culture samples according the protocol of Ahn (2000). 10 μg total RNA were separated on 1.2% formaldehyde agarose gels and blotted to Zeta-probe (BioRad) membranes. The blots were hybridized following the manufacturer's instruction. The probes were amplified by PCR from genomic DNA or reverse transcribed cDNA and labeled by DECAprime II kit (Ambion, Austin, Texas). Signals were quantitated by NIH ImageJ program.

Nuclei isolation and run-on assay

Nuclei were isolated from 10 g Arabidopsis cell culture according the previously described protocol (Yu et al., 1998). The nuclei *in vitro* transcription was carried out using 50 μL nuclei in a 100 μL reaction with 100 mM NH_4SO_4, 5 mM $MgCl_2$, 500 μM ATP, GTP and CTP, 30 μM UTP, 20 μL α-P^{32}UTP (3000 Ci/mmol, 10 μCi/μL, PerkinElmer) and 80 units RNAsin (Promega). The mixture was incubated at 30 °C for 30 min. The reaction was stopped by extracting the reaction using the RNA extraction buffer used in RNA isolation. Subsequently, the labeled RNA was extracted as total RNA isolation. 5 ug linearized plasmid DNAs were blotted onto Zeta-probe membrane using a

BIO DOT apparatus (Bio-rad). The membranes were pre-hybridized in 50% formamide, 7% SDS, 0.25 M NaCl, 0.125 M phosphate buffer, pH 7.2 for 1 hr, then membranes were hybridized at 42 °C in the same solution with $1x10^6$ cpm/mL *in vitro* transcribed RNA overnight. The blots were then sequentially washed with 2X SSC /0.1% SDS, 0.5X SSC/ 0.1% SDS and 0.1X SSC /0.1% SDS. Finally, the blots were wrapped in Parafilm and exposed to the phosphor screen for 48 hr. Signals were quantitated by NIH ImageJ program.

Bombardment of onion epidermal cells

35S::GFP was generated by cloning eGFP coding region into KpnI and BamHI site of pCHF3. 35S::AGP31::eGFP was generated by cloning the eGFP coding region into BamHI site of 35S::AGP31::myc to replace the 6Xmyc fragment. Constructs were bombarded into onion epidermal cell using a Bio-Rad (Hercules, CA) PDS-1000/He particle delivery system as described (Arnim, 2002). After bombardment, the cells were incubated for 18 hr in dark at room temperature before being examined under the microscope.

Transgenic plants and GUS staining

About 1.4 kb up-stream of the translation start site of AGP31 promoter was amplified by PCR from genomic DNA using primers 5'-CCAGAATTCGCAATTACGCTCAAAGTCTCC-3' and 5'-CGCGAGCTCTTTGTTTTGTTTTTGGGTTA-3'
and cloned into the EcoRI and SacI site of binary vector pCHF3 (Borevitz et al., 2000) to replace the 35S promoter in pCHF3. The resulting construct is called P_{AGP31}-pCHF3.

Then, a BamHI-XbaI fragment containing *GUS* coding region from PKYLX71 (Schardl et al., 1987; Lloyd et al., 1992) was cloned into the P_{AGP31}-pCHF3 to generate the construct harboring *AGP31* promoter::GUS (P_{AGP31}::GUS). For 35S::AGP31::myc fusion protein construct, the cDNA of AGP31 was amplified by PCR using primers 5'-CCCGGTACCATGGGTTTCATTGGTAAGA-3'and 5'-TTTGGATCCTTTGGGGCAAGACGG-3' from cDNA to remove the stop codon and cloned into KpnI and BamHI sites of pCHF3, then a fragment encoding 6Xmyc was cloned in-frame to the C-terminus of the AGP31 at BamHI site. *UBQ10* promoter was amplified from genomic DNA using primers 5'-TTTAGATCTGAATTCTGTCCCGACGGTGGTGT-3' and 5'-CGTCCATGGCTTGATCACGGTAGAGAGAATT-3'. The *UBQ10*Promoter:: *Luc*$^{+}$::*AGP31*-3'UTR construct was made by cloning the *UBQ10* promoter and 208 bp 3'-UTR of *AGP31* in front of and behind the luciferase coding region, respectively. All the construct sequences were confirmed by DNA sequencing. The constructs were transformed into plants using the flower dipping method (Clough and Bent, 1998). GUS staining procedure was carried out as described in Bomblies (2000).

Chapter 3 Functional analysis of AGP31

Introduction

AGP31 belongs to a subfamily of non-classical AGP which include TTS1, TT2, HyPRP1, DcAGP1, PvPRP1 and NaPRP4 (Chapter 2). TTS1 and TTS2 can guide the pollen tube growth *in vitro*, but their functions *in vivo* have not been established. These homologs of AGP31 show different expression patterns suggesting they may function in different physiological processes. *NaPRP4*, *TTS1* and *TTS2* are only expressed in flowers. *DcAGP1* transcript is only abundant in the carrot suspension-culture cells. In seedlings *DcAGP1* is only expressed at low levels in the roots. In addition, *DcAGP31* mRNA is neither wound- nor stress-inducible. *AGP31* is highly expressed in roots and flower indicating it may have a distinct function different from its homologs.

Reverse genetics is a strategy to determine a particular gene's function by studying the phenotypes of mutant of the genes of interest. In Arabidopsis, large amounts of T-DNA or transposon tagged insertion lines and their flanking sequences have been integrated in the uniform platform at T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). To identify the mutant of *AGP31*, I queried the T-DNA Express database. Fortunately, there was a transposon tagged line disrupting the AGP31 gene available. Using this mutant line, I applied the reverse genetics to determine the biological significance of AGP31.

Since preliminary observation did not identify any noticeable phenotype of *agp31* mutant growing under normal conditions, I postulated that the either the mutant had a subtle phenotype or there were redundant genes of *AGP31* in the Arabidopsis genome. Based on these two hypotheses, the project was designed to identify the new phenotypes under various growth conditions and to search for the redundant gene(s) of *AGP31*.

To identify the new phenotypes under different growth conditions, I first checked the *AGP31* gene response to various treatments. This kind of experiment may provide some guidance to the mutant phenotype analysis. In addition, I also checked the AGP31 gene expressions in different mutants. This experiment may reveal some regulation pathways for the *AGP31* gene.

Microarray provides a powerful method to monitor the expression of thousands gene simultaneously. To identify differentially expressed genes in the mutant and wild type, microarray technique was applied to wild type and the mutant plants.

Results:

Identification of an AGP31 mutant

To analyze the function of AtAGP31, a transposon insertion line was found in the transposon insertion collection in Landsberg *erecta* from Cold Spring Harbor Laboratory (Fig 2.1A). Primers specific to AGP31 and two ends of Ds insertion fragment were

designed and used to amplify the junctions between the two ends of Ds insertion site and *AGP31* from the genomic DNA. Sequencing the PCR products revealed the insertion site was at 58 bp upstream the stop codon with 8 bp inverted repeat at both end of Ds insertion site. Southern blots using the Ds specific probe proved there was only one Ds insertion in this line (Fig 2.1B).

To confirm that the Ds insertion line knockouts the expression of *AGP31*, RT-PCR using the primers spanned the insertion site shown the mRNA expression of AGP31 was not detectable in transposon insertion line (Fig 2.1 C). Northern blots using the probe upstream the insertion site could not detect any RNA in the transposon insertion line either (Fig 2.1D). To investigate the possibility of partial expression of AGP31, primers just upstream the insertion site were used to do the RT-PCR. A very faint band could be amplified with 36 cycles of PCR (data not shown). Due to the Ds insertion disrupting the polyadenylation signal, the partial transcripts may not be exported to the cytoplasm and form the functional mRNA. Based on these results, I concluded that the DS insertion line is the null mutant of AGP31.

Generation of rescue lines to compliment the agp31 mutant

In order to identify the function of AGP31 unambiguously, rescue lines were generated to test any phenotype showing in mutant agp31. An EcoRI-HindIII fragment from BAC clone F3H6 obtained from The Arabidopsis Information Resource (TAIR) was cloned into the binary vector 35SpBARN (a gift from Dr. Lloyd lab), which carries

the herbicide resistant gene *bar* ($BASTA^r$). This fragment contains 3130bp upstream the translation start codon and 701bp downstream translation stop codon, respectively. Mutant plants were transformed by the flower dip method. The homozygous plants harboring transformed genomic fragments at a single locus were selected through the segregation analysis of herbicide resistance. RNA from 7-day-old seedlings were extracted and subjected to Northern blot analysis. As shown in Figure 2.2, *AGP31* mRNA could be detected in all rescue lines, indicating the successful compliment to the *agp31* mutant. Most rescue lines had higher mRNA levels than wild type plants. This was probably because the multiple copy insertions at the single locus increased the gene dosage. Southern blot analysis indicated all rescue lines had multiple copies of transformed fragments (data not shown). In the later phenotype analysis, the line which showed high expression level (R2) was used to analyze the phenotype of over-expression of AGP31.

AGP31 response to various treatments

Under normal growth condition, *agp31* does not have any visible phenotypes. In order to identify the physiological processes AGP31 involved, *AGP31* gene responses to various treatments were checked by Northern blots. Synthesis of ethylene and oxidative species are common stress responses of plants. *AGP31* gene responses to 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of enthylene biosynthesis), hydrogen peroxide (H_2O_2, mimic the oxidative stress) and NaCl (induce both ethylene biosynthesis and oxidative production) were checked by Northern blot analysis. In

addition, plants were also treated with other phytohormones to investigate their possible roles in *AGP31* regulation. 7-day-old seedlings grown in normal media were transfer to assay plates supplemented with various chemicals to treat the plants for 8 hr. As shown in Figure 2.3, the *AGP31* mRNA could be down-regulated by ACC, NaCl and H_2O_2 treatments while slightly up-regulated by 2,4-dichlorophenoxy acetic acid (2,4D, auxin) treatments. Gibberellin A_3 (GA_3) and 6-benzylaminopurine (BAP, cytokinin) had no effect on the *AGP31* expression. Since all of these treatments representing stresses (MeJA [Chapter 2], ABA [Chapter 2], ACC, H_2O_2 and NaCl) could down-regulated *AGP31* mRNA, I concluded that the *AGP31* gene was down-regulated during stress responses.

AGP31 expression levels in different mutants

In order to identify possible genes regulating AGP31, I checked the expression patterns of *AGP31* in different mutant backgrounds. Since JA and ABA are the major stress regulating phytohormones, I chose the representative mutants involved in JA and ABA signaling and synthesis pathways. As shown in Figure 2.4, ABA deficient mutants (*aba1-1* and *aba3-2*) had the highest *AGP31* expression levels. ABA signaling mutants (*abi1-1* and *abi2-1*) and JA biosynthesis mutant (*aos*) also had slightly higher expression levels than wild type plants. It is noteworthy that JA biosynthesis knockdown mutant 35S::LOX2 didn't show higher expression level than wild type plants. Since lipoxygenases (LOXs) catalyze the conversion of linolenic acid to 13-hydroperoxylinolenic which is the sencond step for jasmonate biosynthesis, there might

be redundant genes or pathways for jasmonate biosynthesis. Knockdown LOX2 may not reduce the jasmonate biosynthesis sufficiently.

To investigate if the JA or ABA signaling pathways are involved in the *AGP31* stress responses, JA and ABA signaling mutants *coi1-1*,*abi1-1*,*abi2-1* and *abi3-1* were used to check the *AGP31* response upon JA and ABA stress treatments. As shown in Figure 2.5, except for *coi1-1* which alleviated the reduction of *AGP31* to JA treatment as shown in chapter 2, no other mutants were involved in the *AGP31* repression to JA and ABA treatment.

Phenotype analyses of *agp31* mutant under stress responses

Since stress treatments can repress the *AGP31* gene expression, it is possible *agp31* mutant may interfere with some stress responses. Extensive phenotype analyses were carried out to identify possible phenotype in *agp31* mutant grown under various stress conditions. The wild type and rescue plants were tested at the same time with the mutant. Unfortunately, there were no significant differences between mutant and wild type plants under all the conditions tested. The conditions tested include temperature (high 37 °C or low 4 °C), light (dark or light), water supply, various concentrations of phytohormone (2,4 D, kinetin, BAP, ACC, GA3, JA, ABA), various concentrations of plant macronutrients (N, K, P, Ca, Mg), various concentrations of plant micronutrients (B, Cu, Mn, Fe, Zn), heavy metal stress (Cd, Ar) and proline toxicity.

Microarray analysis to identify the differentially expressed genes in *agp31* and wild type plants

Since no visible phenotype could be identify, microarray was used to identify the differentially expressed genes in *agp31* mutant and wild type plants. I used 70-mer oligonucleotide microarrays manufactured by the University of Arizona, which contains 29,000 elements, to analyze the gene expression difference between mutant and wild type plants grown under normal conditions. SAM (Significance Analysis of Microarrays) analysis reveals there are 193 and 17 genes up-regulated and down-regulated in mutant plants, respectively at 2.2 FDR (False Discovery Rate) level. These genes are the preliminary differentially expressed genes in *agp31* (PDEG). To identify the differentially expressed genes which are due to the mutation in gene *AGP31*, mRNA from normal growing wild type and one rescue line plants were isolated and subjected to microarray analysis. In this microarray analysis, any genes still showing differentially expression pattern were most likely caused by transposon footprint and were removed from PDEG. After this operation, there were 56 up-regulated and 6 down-regulated genes left in the PDEG and these genes were considered as the real differentially expressed genes (DEG) due to *AGP31* mutation. To simplify the analysis, I just chose the genes which showed at least 2 fold change (either increase or decrease 2 fold) to do the northern blot confirmation. Surprisingly there were only 3 genes that passed these criteria. These genes were Cruciferin 1 (CRA1), Cuciferin 3 (CRU3) and Oleosin 2 (OLE2). All of these genes were up-regulated in mutant plants. The northern blot analyses confirmed the expression patterns revealed by microarray analyses (Fig. 2.6).

Seed germination assay of mutant plants

Since the mutant of gene *AGP30* (At2g33790), which has high similarity with AGP31 except without poly-histidine rich motif, had been shown resistant to the ABA inhibition of seed germination, I tested the germination rate of *agp31* seeds under ABA treatments. Seeds were first imbibed at 4 °C for 3 days in dark to break the dormancy and then transferred to light at 22 °C to start the germination. Seed germinations were scored as radicle protrusion 6 days after germination. As shown in Figure 2.7, *agp31* seeds were less sensitive to the ABA inhibition. To test whether the ABA resistance was due to the *AGP31* mutation, seeds of the rescue plants (R2, R16) were tested at the same conditions. But the rescue plants could not complement the ABA resistance phenotype of *agp31* mutant.

AGP31 are expressed in the phloem in inflorescence stem and embryo vascular bundle

Since *agp31* affects the seed storage protein expressions, I checked their expression patterns in seeds and inflorescence stems. AGP31Promoter::GUS transgenic plants were generated (described in chapter 2). GUS staining revealed that *AGP31* was expressed in vascular bundles of embryo cotyledons (Fig. 2.8, A and B) and in the phloem regions of inflorescence stems (Fig. 2.8, C and D). The expression patterns of *AGP31* in vascular bundles of embryos and inflorescence stems implied they might function in the phloem transportation to regulate the seed storage protein expressions.

Discussion

In this chapter, I described that knockout a cell wall structural protein gene, *AGP31*, could induce the expressions of some seed storage proteins. To my knowledge, this was the first time to show a cell wall structural protein had such kind function. Seed storage proteins have import impact on the nutritional quality of human and livestock food. The discovery here may provide an alternative method to manipulate the seed storage protein in important economical crops.

During late embryo development, seeds accumulate large amounts of storage proteins (SSP) which are degraded during seed germination to serve as an amino acid supply. In Arabidopsis the major SSP are 12S globulins (also called cruciferin in Arabidopsis) and 2S albumins (also called napin in Arabidopsis) classified according to their solubity in high salt solution or water, respectively. 12S globulin is a hexameric complexes consisting of six subunits. Each subunit is composed of one α and one β polypeptide linked via disulfide bonds. The α and β polypeptides are synthesized from a single polypeptide-precursor by cleavage. 2S albumin is a heterodimer consisting of 2 peptides linked by disulfide bonds. Similarly, the 2S albumin is also proteolyticaly synthesized from a single polypeptide procursor (Fujiwara et al., 2002).

In Arabidopsis, both the 12S globulins and 2S albumins are encoded by a small gene family. There are 3 genes encoding 12S globulin in Columbia ecotype. In

Landsberg ecotype there is another gene encoding 12S globulin. Five genes encoding 2S albumin named At2S1 to At2S5. At2S1 to At2S4 are located in chromosome 4 as a tandem cluster (Fujiwara et al., 2002).

The expression of SSP genes are under tight temporal and spatial control. SSP genes are strictly expressed in the mid to late embryo development stage. Consequently, the expression of SSP genes is considered an important characteristic of seed maturation. The mutants of SSP gene have not been reported probably due to gene redundancy causing lacking of phenotypes in any single mutant. Vast efforts have been invested to dissect the regulation mechanism of SSP gene expression and substantial progresses have been made. A number of cis-elements in the SSP gene promoters and the trans-element which bind them have been identified. In Arabidopsis, the cis-elements identified so far are RY motif (CATGCA) and ACGT motif which are the binding targets of B3 and bZIP transcription factors, respectively (Ellerstrom et al., 1996; Ezcurra et al., 1999; Ezcurra et al., 2000; Nakabayashi et al., 2005; Suzuki et al., 2005). There are three B3 transcription factors ABI3 which regulates the SSP gene expression indirectly, LEC2 and FUSCA3 which directly bind the RY region of Arabidopsis SSP promoters, that positively regulate the expression of SSP genes during seed maturation stage (Kroj et al., 2003). The bZIP transcription factors regulating the SSP gene expression are AtZIP10 and AtZIP25 which are the homologs of OPAQUE2 (O2) (Lara et al., 2003). O2 is the transcription factor originally identified in maize and regulates the maize SSP zein expression. The O2 homologs are widely found in monocot crops such as wheat (SPA) (Albani et al., 1997),

rice (RISBZ1 and RITA1) (Izawa et al., 1994; Onodera et al., 2001), barley (BLZ1 and BLZ2) (Vicente-Carbajosa et al., 1998; Onate et al., 1999), sorghum (Pirovano et al., 1994) and coix (Vettore et al., 1998) and regulate their SSP gene expression correspondingly. In Arabidopsis, AtZIP10 and AtZIP25 act synergistically with B3 transcription factor ABI3 in the activation of SSP gene expression as demonstrated by the analysis of At2S1 promoter activity in plants ectopically expressing AtZIP10, ATZIP25 and ABI3 individually or combinatorially (Lara et al., 2003).

Other transcritption factors involved in the SSP gene expressed are *LEAFY COTYLEDON 1* (*LEC1*) and *LEC1-LIKE* (*L1L*) which belong to the HAP3 family CCAAT-binding transcription factors. LEC1 control the seed storage proteins through FUSCA3 and ABI3 (Kagaya et al., 2005). L1L has distinct function from LEC1 during seed development but ectopically express L1L can complement the *lec1* mutant (Kwong et al., 2003).

In addition specifically activating the SSP gene expression during seed maturation stage, plants need to suppress their expressions during vegetative growth stage. HSI2 and HSL1—another two B3 transcription factors, act as the repressors of SSP gene expression in early seedling stage. *hsi2* and *hsl1* double mutant plants showed the vegetative expression of SSP At2S3 (Tsukagoshi et al., 2007). It has been shown that several transcription factor VAL (VP1/ABI3 like) genes are required for the suppression

of embryonic traits (including seed storage protein genes) during seedling stage (Suzuki et al., 2007).

During seed maturation, besides accumulation of nutritional seed storage proteins, plants also accumulate other kind storage proteins such as oleosins and late embryo proteins (LEAs). Oleosins are small hydrophobic proteins proposed to function in maintaining the structural integrity of oil bodies and serving as a recognition signal for lipase binding during oil mobilization in seedlings (Lee et al., 1991; Siloto et al., 2006). Some genes regulating the globulins and albumins such as VALs also regulate the expression of some oleosins (Suzuki et al., 2007) .

In this dissertation, I showed that knockout of a cell wall protein, AGP31, induced the expression of several SSP genes (*CRU3*, *CRA1* and *OLEOSIN2*) in vegetative tissue. More importantly, the SSP gene subset induced by AGP31 knockout is different from those of known SSP regulators. To my knowledge, this was the first time to show knock-out of a cell wall structural protein could induce the expression of seed storage proteins. Knock-out of *AGP31* induced a new set of SSPs suggesting there was a new pathway to regulate the expressions of *CRU3*, *CRA1* and *OLEOSIN2*. This hypothesis is supported by several pieces of evidences. First, in Arabidopsis all known genes regulating SSP gene expressions identified so far are transcription factors. Compared to *agp31* mutant, mutations or ecotopic expressions of these genes cause the expression of different sets of SSP gene to be altered in seedlings (Kagaya et al., 2005; Kagaya et al., 2005; Santos Mendoza et al., 2005; Braybrook et al., 2006). The selective induction of specific SSPs

by *AGP31* knockout suggests that there are additional pathways or transcription factors targeting these SSP genes. Second, microarray analysis of *agp31* mutant did not identify any expression changes of the transcription factors which are known to regulate SSP gene expressions. This is consistent with the hypotheses that these transcription factors are not involved in the induction of SSPs by *agp31* knockout.

The expressions of SSP during the seed maturation are also regulated by phytohormone ABA. The activation of SSP gene expression of many transcription factors are ABA dependent. For example, activation SSP expressions by ABI3, FUSCA3 are ABA dependent (Suzuki et al., 2001; Kagaya et al., 2005). AGP31 mRNA is down-regulated by exogenous ABA application while up-regulated in ABA deficient mutants presumably due to lower ABA contents in these mutants. The expression pattern of AGP31 regulated by ABA might correlate the expression pattern of seed storage proteins induced by *agp31* to ABA signaling pathways.

Another phytohormone regulating seed maturation and germination is gibberellin (GA) which promotes the seed germination. During seed maturation, FUSCA3 and LEC2 repress the expression of AtGA3ox2, which encodes the key enzyme that catalyzes the conversion of inactive GAs to bioactive GAs (Curaba et al., 2004). During seed germination, both endogenous active GAs and AtGA2ox2 mRNA levels increase (Ogawa et al., 2003). Mutations in GA biosynthesis or application of GA biosynthesis inhibitor inhibit the seed germnation. PICKEL, a CHD3 chromatin remodeling factor mediating a subset of GA responses, suppresses the embryo traits in seedlings (Ogas et al., 1999). Mutation *pickle* causes the seedling to exhibit embryonic traits, including accumulation

of seed storage reserves (oil, protein and phytate) and the ability to undergo somatic embryogenesis (Ogas et al., 1997; Rider et al., 2004; Li et al., 2005). Interestingly, not all embryo trait suppressions in seedlings are PICKLE dependent (Dean Rider et al., 2003; Rider et al., 2004). Microarray analysis of *agp31* did not detect the reduced expression of PICKLE gene implying that PICKLE was not involved in the SSP expression in *agp31* mutant. This observation corroborates the hypothesis that there are other unidentified pathways to suppress the expression of some seed storage proteins.

It is intriguing that the knockout of a cell wall protein, *agp31*, can induce some seed storage proteins. Several other cell wall protein genes, atGRP3 (Glycine rich protein 3) and FLA7 (fasciclin-like arabinogalactan-protein 7), has been observed up-regulated among other genes in *pickle* mutant and proposed as the candidates of PICKLE dependent regulators of embryo traits (Dean Rider et al., 2003). I postulate that the function mechanism of these cell wall proteins might be that they control the nutrition flow to the cell by adjusting the cell wall structure. Nutrition supply has been shown to affect the seed reserves accumulation. Limiting sulfur nutrition has also been found to increase certain SSP accumulations while decreasing other SSP accumulations (Tabe and Droux, 2001, 2002; Higashi et al., 2006). Amino acid and sugar supply play crucial role in the accumulation process of seed reserves. In maize, the absence of alpha-zein in the endosperm of opaque2 (o2) mutant can be recovered by amino acid supply (Locatelli et al., 2001). Amino acid transporters, AAP1 expressed in endosperm and cotyledons and AAP2 expressed in the phloem of stems and veins supplying seeds, have implicated to be

involved in long distance amino acid transportation to seeds (Hirner et al., 1998). Glucose transporter AtVGT1 (vacuolar glucose transporter 1) has been shown to play an important role in seed germination and plant flowering (Aluri and Buttner, 2007). Sporamin, the tuberous root storage protein of the sweet potato, can be induced by sucrose, glucose and fructose (Hattori et al., 1990; Nakamura et al., 1991). HSI2 and HSL1, the repressors of the sporamin promoter, are also required for repression of the SSP gene expression in vegetative tissue in Arabidopsis (Tsukagoshi et al., 2007).

The mutant of *agp31* is insentitive to the ABA inhibition of seed germination, but this phenotype cannot be complemented by introducing the AGP31 gene back into the mutant. Originally this was ascribed to the footprints of transposon which might disrupt some other genes during transposon mutagenesis. However, due to the knockout of agp31 induces the expression of SSP and AGP 31 mRNA is regulated by endogenous ABA level, it is possible that the ABA insensitivity of *agp31* seeds may be the true phenotype. A possible explanation for insensitivity of rescued lines is that the over-expression level of AGP31 gene in the rescued lines may saturate the glycosylation system for AGP31. These under/non glycosylated AGP31 are exported to the cell wall and function as a competitor to the fully glycosylated AGP31. Further experiments are needed to clarify this hypothesis.

There are several possible mechanisms that *agp31* induces the expression of seed storage protein. First, AGP31 may function as a cell wall structure protein. Mutation in

this gene affects the cell wall structures such that the permeability of certain molecules is altered. The availability of these molecules may modulate the expression of seed storage proteins. Second, AGP31 can function as a co-transporter of some molecules. Similarly, the availability of these molecules may affect the seed storage protein expressions. The molecules AGP31 affected can be phytohormones such as ABA or JA, nutritional molecules (such as amino acid), oxygen and carbon dioxide.

Materials and Methods

Materials and growth conditions

The wild type used here is Ler-0. AGP31 plants were obtained from Cold Spring Harbor Laboratory Transposon genetrap line GT6134. The plants were grown under continuous light at 22 °C. Seedlings are grown in half MS 1% sucrose in Petri dishes. Plants were grown to maturity in Metromix 200 soil. For plant chemical treatment experiments, 7-day-old plants grown on plates (half MS media with 0.8% agar) were transferred to assay plates (same media as growing plates supplemented with indicated chemicals) for 8 hr. For germination assay, seeds were sowed on half MS media with 0.8% agar plates. Seeds were stratified at 4 °C for 72 hr and transferred to light at 22 °C to start germination. Germination ratios were counted by the radical protusion.

Mutant identification

Search of the T-DNA express database reveal there is one transposon genetrap line GT6134 harboring a transposon in AGP31 gene. The seeds were obtained from Cold

Spring Harbor Laboratory. Thirty plants were chosen to subject genotype analysis by PCR. Genomic DNA was extracted from one piece of the leaf of every individual plant as described previous (Dellaporta et al., 1983). Primer pair 5'-GGCTCTGCATACGCATTCAAACAG -3' and 5'-CAAACGAAACTCAAACGACGTCAC-3' were used to identify the wild type locus. Primer pair 5'-GGCTCTGCATACGCATTCAAACAG -3' and 5'-TGAAACGGTCGGGAAACTAGCTCT-3' were used to identify the transposon insertion locus. Plants showing homozygous locus were kept to set seeds. Seeds were tested for their kanamycin resistance in the half MS agar plates containing 50 μg/mL kanamycin resistance (50 μg/mL). Homozygous plants were confirmed by their 100% resistance to kanamycin. Primer pairs G2M-F: 5'-GGCTCTGCATACGCATTCAAACAG -3', Ds5-R: 5'-TGAAACGGTCGGGAAACTAGCTCT-3' and Ds3-F: 5'-TTTCTTGTAACGCGCTTTCCCACC -3', G2M-R: 5'-CAAACGAAACTCAAACGACGTCAC-3' were used to amplify the junctions between transposon and genomic DNA, the PCR products were cloned into Topo-PCR (Invitrogen) vector to sequence the junctions.

Northern and Southern blot

Nothern blots were carried out as outlined in Chapter 2. For Southern blot analyses, 2 μg genomic DNA isolated by Plant DNAZol (Invitrogen) was digested by

indicated enzymes and transferred to Zeta-probes (Bio-rad) by alkaline transfer buffer. Hybridization was carried out using the same protocol as in Northern blots.

RT-PCR

For RT-PCR to analyze the *agp31* expression, total RNA was isolated from 7-day-old seedlings by Trizol method. Total RNA was digested by Turbo DNAse (Invitrogen) to eliminate genomic DNA contamination. 1 μg DNase treated RNA were reverse transcribed to cDNA by MMLV reverse transcriptase (Invitrogen) in 20 uL reactions. 2 μL cDNA were used to amplify the designated targets.

Microarray analysis

The 70-mer oligonucleotide microarrays (University of Arizona) were used to carry out microarray analysis. Total RNA were isolated from 7-day-old seedlings using Trizol method. mRNA were isolated using Oligotex mRNA isolation kit (QIAGEN) following the instruction of manufacturer. cDNA were reverse-transcribed and labeled by Cy3 and Cy5 dye by indirect labeling procedure by CyScribe cDNA Post Labelling Kit (Amersham) following the manufacturer's instruction. The slide hybridizations were carried out by following the protocol supplied with the slides (http://ag.arizona.edu/microarray/methods.html). The slides were scanned by Genepix 6000B. Data extraction and quality control were done by Genepix 6.0. Data were analyzed by BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html).

Significance Analysis of Microarrays (SAM) were carried out by using excel plug-in (http://www-stat.stanford.edu/~tibs/SAM/). Three independent biological replicates were subjected to microarray analyses for wild-type and mutant comparison. One replicate was used for wild-type and rescue line plants.

Chapter 4 Future Works

This dissertation established the function of a non-classical AGP—AGP31. Using this system, it is possible to discover the molecular mechanism of AGP31 function. Some very promising directions resulting from these studies are:

1. Since AGP31 can be isolated from plants without removing their oligosaccharide chains, it is an excellent model to discover the glycosylation pattern of AGP. Glyco-side chain analysis should reveal the sugar linkage of AGP31. By manipulating the coding sequence of AGP31, this system can be used to validate the Hyp continuity hypothesis which predicts that the contiguous Hyp residues are typically arabinosylated, whereas noncontiguous clustered Hyp are typically arabinogalactosylated.

2. The selective repression of certain SSP gene by AGP31 represents a new pathway of SSP gene regulation. Screen the suppressor of agp31 mutant should reveal the down-stream component of this pathway.

3. Using different motifs to complement the agp31 mutant should reveal the function of every motif in AGP31, especially the function of the non-proline rich region

(PAC domain). The non-proline rich region has been found in a number of AGP from different species and implicated in protein-protein interaction.

4. Since most AGPs' functions are unknown, double mutant, triple mutant, quadruple mutant of different AGPs can be made to test the functions of a certain type of AGPs.

5. The functions of AG peptides are completely unknown, it is very interesting to extend similar analysis described in this dissertation to AG peptide genes.

6. More precise bioinformatics methods need to be designed to identify the non-classical AGPs in Arabidopsis genome.

Amino acid	Experimentally determined composition (Mole%)	Predicted composition (Mole%)
Hyp	14.5	
Pro	13.1	26.9
Lys	14.0	14.9
Val	11.4	12.2
Ala	6.6	6.6
Thr	6.0	6.9
His	5.6	4.5
Gly	5.4	3.9
Glu	4.7	1.8
Ser	3.8	4.2
Tyr	3.7	3.9
Leu	3.6	4.2
Asp	3.5	0.9
Phe	2.0	2.7
Arg	1.2	1.2
Ile	0.9	0.6
Met	0.1	0
Cys	NA	4.5

Table 1. 1 The amino acid composition of AGP31

Glycosyl residue	Mole %
Galactose	80.9
Arabinose	13.5
Xylose	2.1
Fucose	1.3
Mannose	1.3
Glucuronic acid	0.5
Glucose	0.5

Table 1. 2 Monosaccharide composition of AGP31

A

*MGFIGKSVLVSLVALWCFTSSVFT*EEVNHKTQTPSLAPAPAPY**H**
HGHHHPHPPHHHHPHPHPHPHPPAKSPVKPPVKAPVSPPAKPPV
KPPVYPPTKAPVKPPTKPPVKPPVSPPAKPPVKPPVYPPTKAPV
KPPTKPPVKPPVYPPTKAPVKPPTKPPVKPPVYPPTKAPVKPPT
KPPVKPPVSPPAKPPVKPPVYPPTKAPVKPPVSPPTKPPVTPPV
YPPKFNRSLVAVRGTVYCKSCKYAAFNTLLGAKPIEGATVKLVC
KSKKNITAETTTDKNGYFLLLAPKTVTNFGFRGCRVYLVKSKDY
KCSKVSKLFGGDVGAELKPEKKLGKSTVVVNKLVYGLFNVGPFA
FNPSCPK

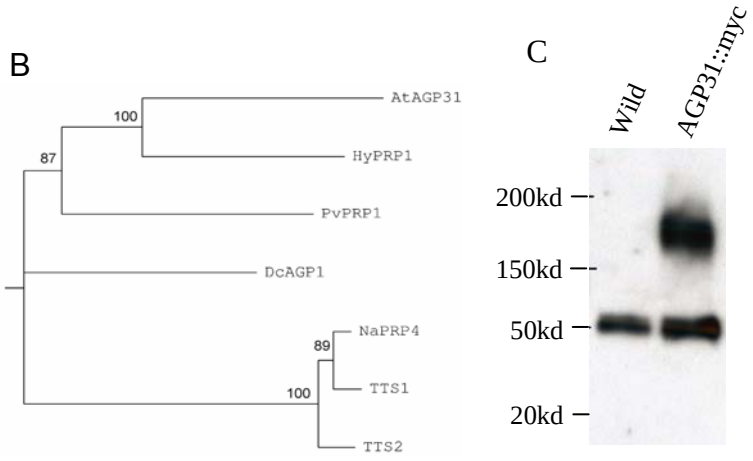


Figure 1.1 Characterization of AGP31.

A. The deduced amino acid sequence of AGP31. Shown are signal peptide (italics), predicted arabinogalactanylation and N-glycosylation sites (pink), his-rich region (bold), four exact repeat units (red and blue), and two larger exact repeat units (underlined). The smallest repeat unit is XXPP. B. Neighbor joining tree of the homologs of AGP31, numbers are bootstrap number expressed as percentage in 1000 bootstrap replicates. C. Western blot of cell wall AGP precipitate from AGP31:myc expressing plants, probed with anti-myc antibody.

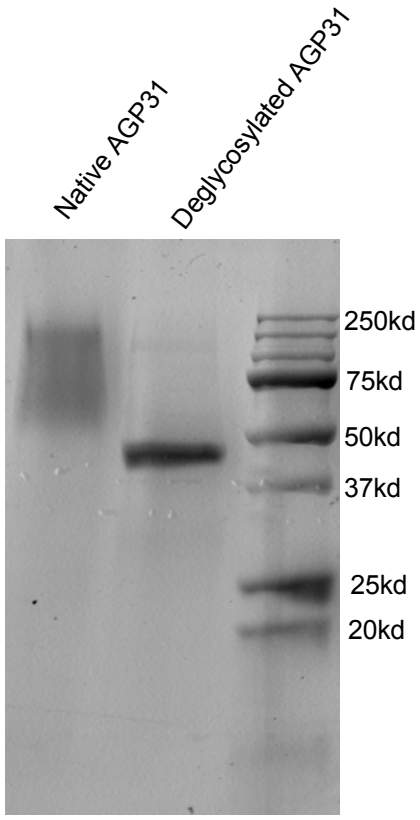


Figure 1.2 Deglycosylated AGP31

AGP31 was isolated from high salt eluted cell wall protein by NTA-Ni column and deglycosylated by TFMS. Approximately 2 μg of native and deglycosylated AGP31 were run by SDS-PAGE in 15% Tris-glycine gel. Gel was stained by Commassie blue

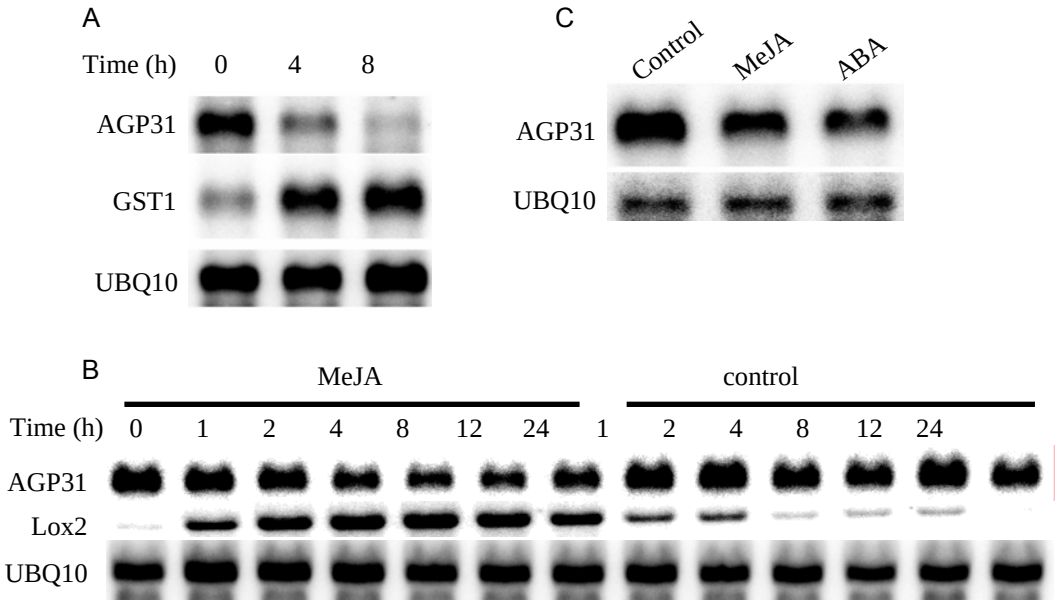


Figure 1.3 Effects of wounding, MeJA and ABA treatments on *AGP31* and reference mRNA levels.

A. Northern blot analysis of RNA from wounded leaves of 4-week-old plants. About 1/3 of the leaves were pressed with a pliers and wounded leaves were harvested at given time points.

B. Northern blot analysis of RNA from MeJA treated 7-day-old plants. Seedlings were grown in Petri dishes, sprayed with 500 μM MeJA in 0.1% ethanol, 0.01% tween 20 or solvent control (0.1% ethanol, 0.01% tween 20)., and whole seedlings were harvested at given time points.

C. Northern blot analysis of RNA from ABA treated 7-day-old plants. Seedlings were grown in Petri dishes, transferred to assay plates containing 0.1% ethanol solvent control, 50 μ M MeJA and 10 μ M ABA for 8 hours, and whole seedlings were harvested at given time points.

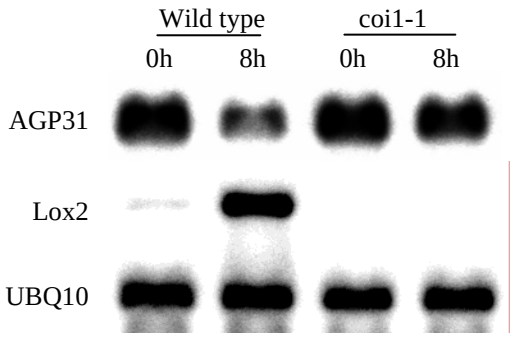


Figure 1.4 Effects of MeJA treatment on *AGP31*, *Lox2* and *UBQ10* mRNA levels in wild type and coi1-1 plants.

Northern blot analysis of RNA from 7-day-old plants, either wild type or coi1-1, treated with 50μM MeJA for 0 and 8 hr. The same blot hybridized with the *AGP31* probe was washed and rehybridized with *Lox2* and then again with *UBQ10* probe.

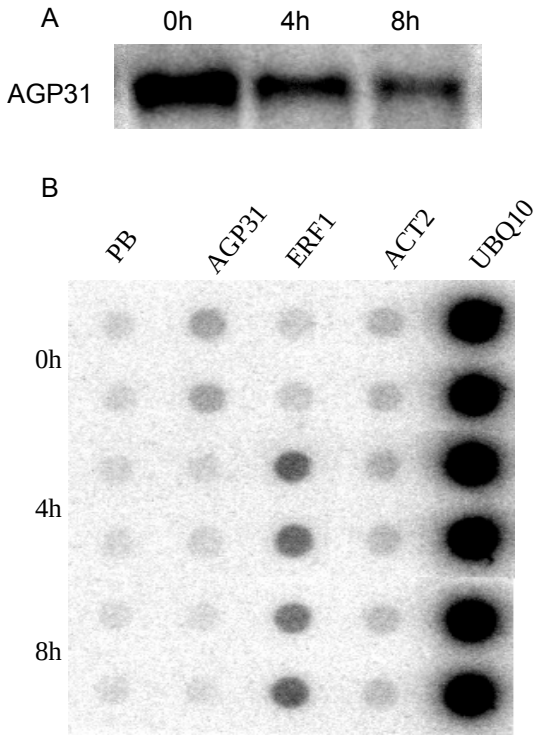


Figure1.5 mRNA steady state levels and transcription rates of different genes in Arabidopsis cultured cells treated with MeJA.

A. Northern blot analysis of *AGP31* mRNA in MeJA treated (50 μM) cultured cells harvested at the given time points.

B. Nuclei were isolated from the same batches of one week cultured Arabidopsis suspension cells as shown in A at the given time points. After incubation with ^{32}P-UTP to produce radiolabeled run-off transcripts, RNA was purified from nuclei and hybridized to duplicate spots for each plasmid DNA: PB, pBluescript vector; ERF1, ethylene response factor (a known MeJA-induced gene); *ACT2, actin 2; UBQ10*, *ubiquitin 10*.

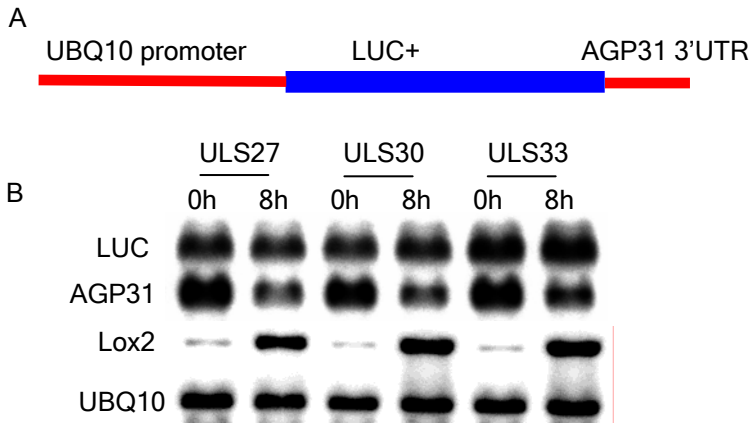


Figure 1.6 The effect of MeJA on the levels of *luc*⁺ reporter mRNA containing the 3'UTR of *AGP31* in transgenic seedlings.

A. The construct structure of UBQ10 promoter-LUC⁺-AGP31 3'-UTR gene.

B. Northern blot analysis of RNA from transgenic plants carrying a *UBQ10 promoter-LUC⁺-AGP31* 3'UTR construct. 7-day-old seedlings of 3 single insertion lines were sprayed with 500 μM MeJA and harvested at the given times. The same blot was sequentially hybridized to the following probes: *LUC⁺*, *AGP31*(to detect endogenous *AGP31* mRNA), *Lox2*, and *UBQ10*.

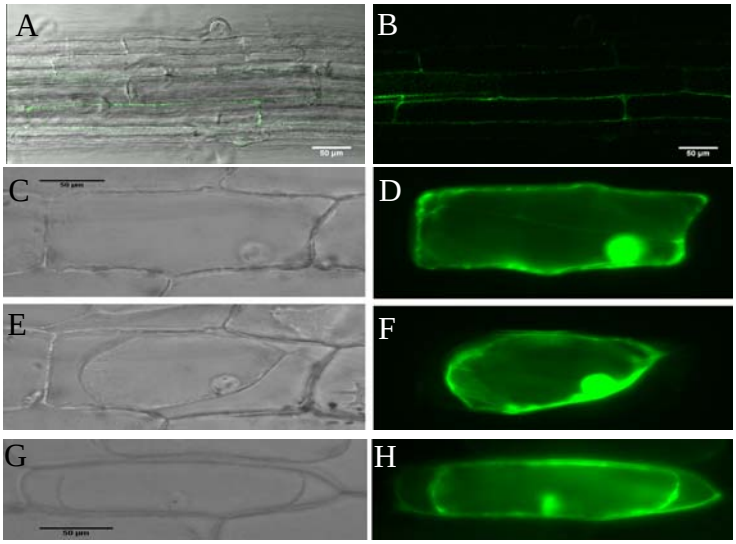


Figure 1.7 Subcellular localization of AGP31-GFP fusion protein.

The eGFP was fused with the C-terminus of AGP31and stably transformed into Arabidopsis (A,B) or introduced biolistically into onion epidermal cells (G,H). The control is eGFP alone introduced into onion biolistically (C—F). C, D unplasmolyzed cell. E—H plasmolyzed cells. The indicated bar scales are used for A and B, C-F and G and H.

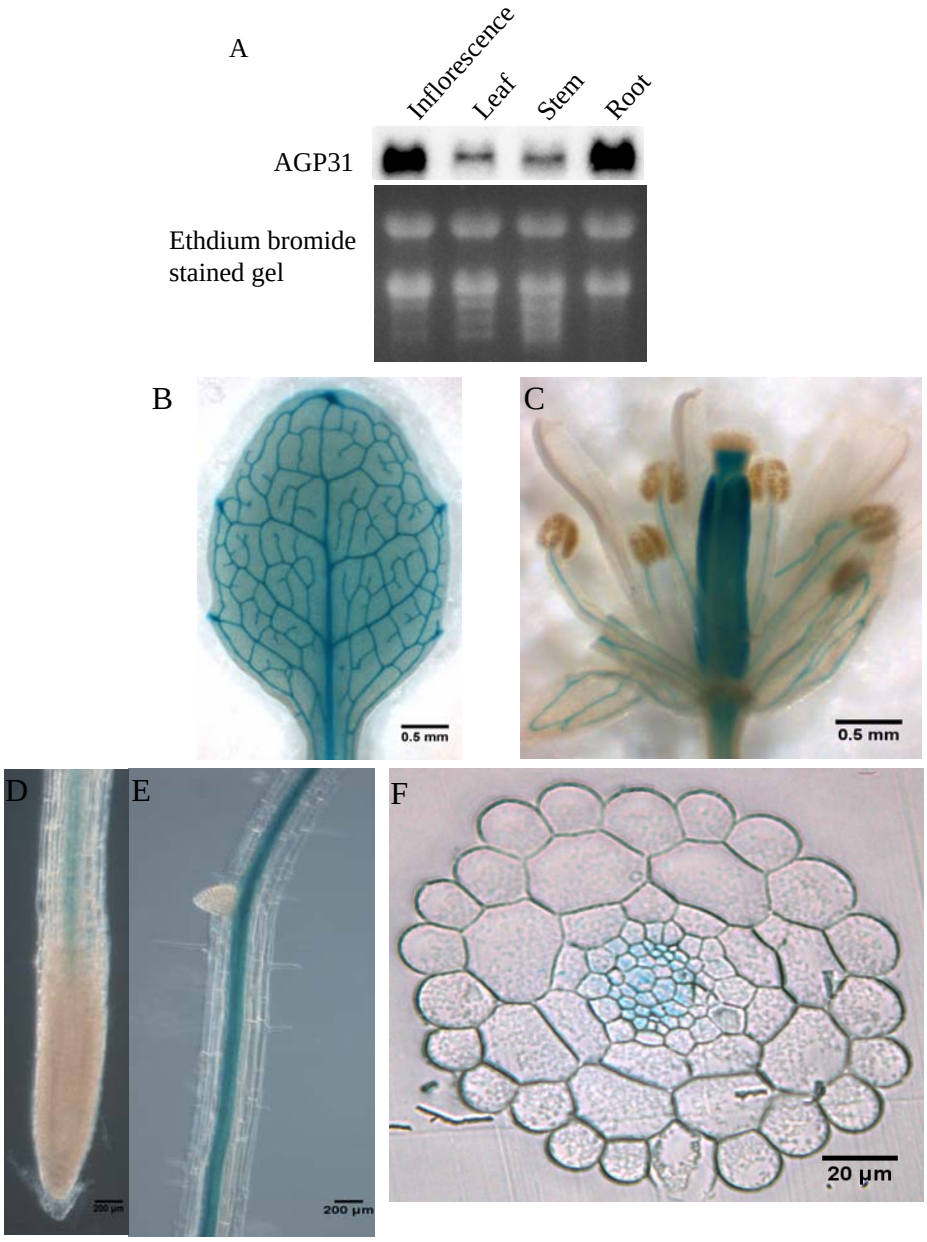


Figure 1.8 GUS staining of transgenic plants harboring the *AGP31* upstream 1.4 kb sequence-*GUS* reporter construct.
An approximately 1.4kb genomic fragment upstream of the *AGP31* ATG start codon was fused with *GUS* reporter gene and transformed into wild type Arabidopsis. A: Northern blot of *AGP31* mRNA in different plant parts B: leaf, C: a flower in stage 15-16. D and E: root. F: cross section of root.

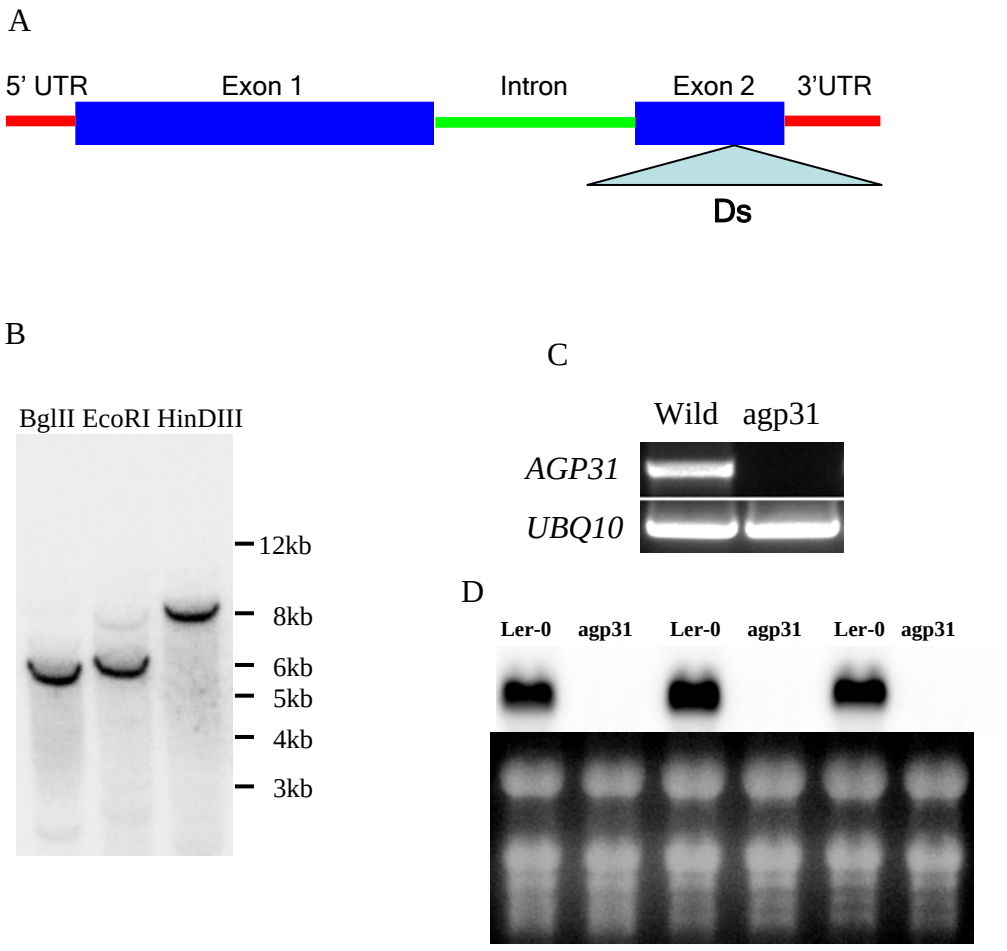


Figure 2.1 Identification of *agp31* mutant

A. The structure of *AGP31* and the position of transposon insertion

B. Southern blot using Ds probe confirms the single insertion of transposon

C. *AGP31* mRNA was undetectable by RT-PCR *agp31*

D. *AGP31* mRNA was undetectable by Northern blot in *agp31*

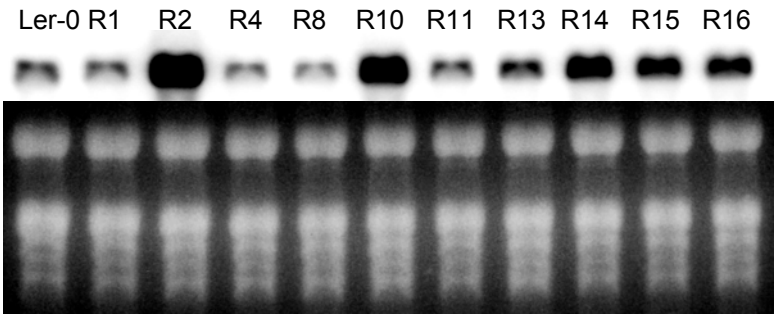


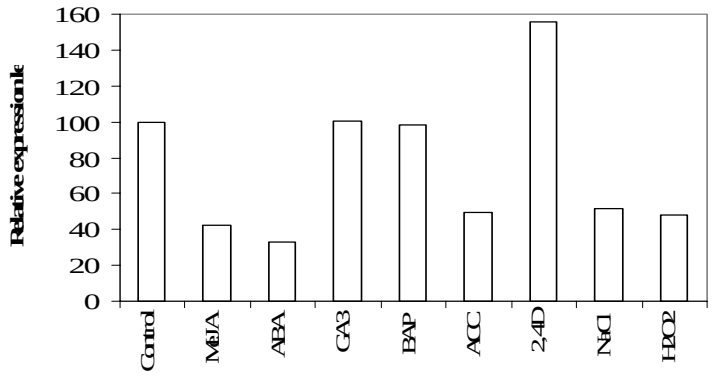
Figure 2.2 Nothern blot analysis to check the *AGP31* gene expression level in rescued lines

A 5.3 kb EcoRI-HindIII fragment containing 3130bp upstream the translation start codon and 701bp downstream translation stop codon, respectively, was cloned into the binary vector 35SpBARN (a gift from Dr. Lloyd lab) which carries a herbicide resistant gene *bar* (BASTA^r) and transformed into *agp31* mutant plants. Homozygous plants harboring single locus T-DNA insertions were selected by following the herbicide resistance segregation during 3 generations. RNA was extracted from 7-day-old seedling growing in half MS agar plates and subjected to Northern blot analysis.

Ler-0: wild type plants

R#: independent rescue lines

A



B

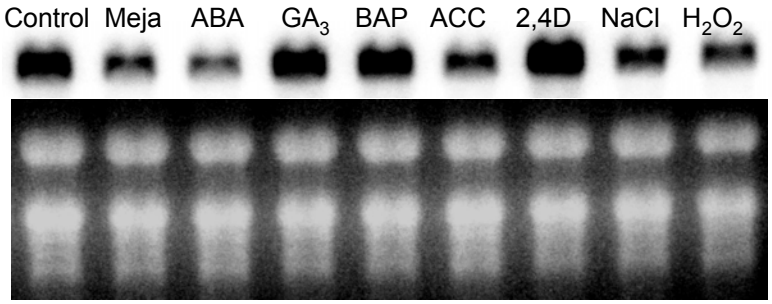


Figure 2.3 *AGP31* gene response to various treatments in wildtype plants

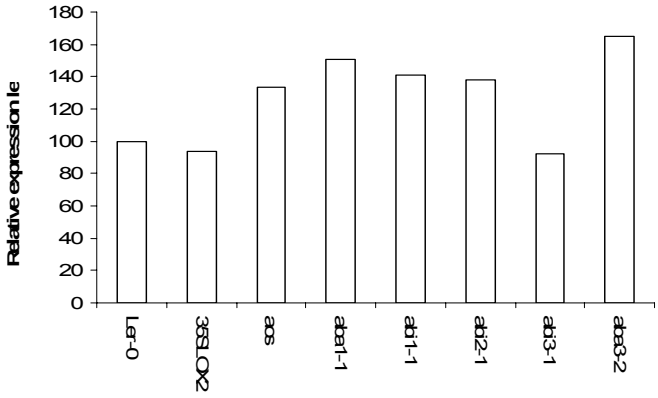
The plants were treated with indicated chemicals by transferring 7-day-old seedlings growing in half MS to the plates supplemented with indicated chemicals for 8 hours. After treatment, whole seedlings were frozen in liquid nitrogen and RNA were extracted by Trizol method.

A: quantification of Northern blot in B. Values are given relative to 100% control sample

B: Nothern blot hybridized with *AGP31* probe

Control: 0.1% Ethanol solvent control, MeJA: 50μM, ABA: 10 μM, GA3: 50 μM, BAP: 10μM, ACC: 20 μM, 2,4 D: 1 μM, NaCl: 150 mM, H2O2: 5 mM.

A



B

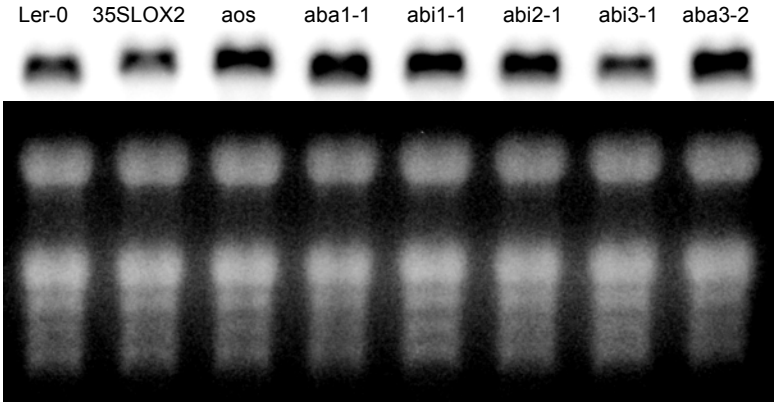


Figure 2.4 *AGP31* gene expression in some MeJA and ABA signalling and biosynthesis mutants

RNA was extracted from 7-day-old seedling growing in half MS agar plates and subjected to Northern blot analysis.

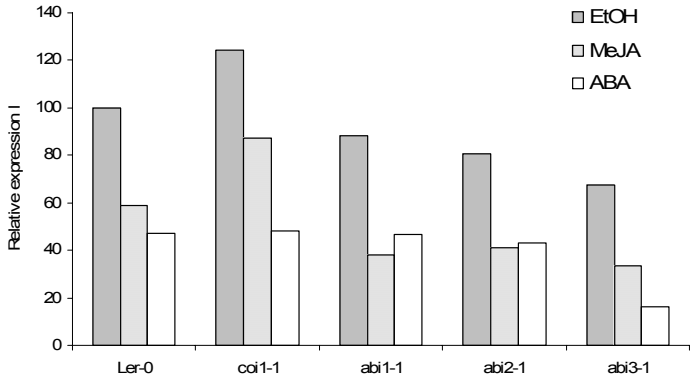
A: quantification of Northern blot in B. Values are given relative to 100% Ler-0

B: Nothern blot hybridized with *AGP31* probe

Ler-0: wild type plants,

35SLOX2: transgenic plant harboring 35SLOX2 constructs which caused reduced expression of lipoxygenase 2 gene. aos: mutant of allene oxide synthase (AOS). aba1-1: abscisic acid deficient; mutant of a single copy gene of zeaxanthin epoxidase. aba3-2: abscisic acid deficient; mutant of molybdenum cofactor sulfurase. abi1-1: ABA insensitive mutant. abi2-1: ABA insensitive mutant. abi3-1: ABA insensitive mutant.

A



B

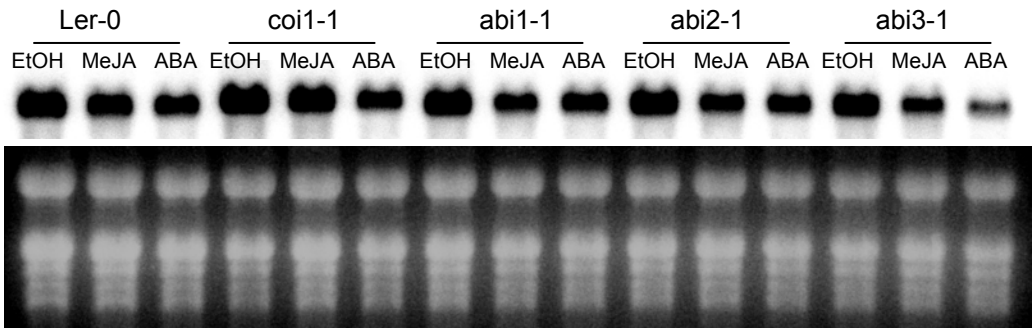


Figure 2.5 *AGP31* gene response to MeJA and ABA treatments in some JA and ABA signaling mutants

7-day-old seedling growing in half MS agar plates were transferred to EtOH (0.1% Ethanol, solvent control), MeJA (50 μM) and ABA (10 μM) assay plated for 8 hours. After treatments, plants were frozen in liquid nitrogen and RNA were extracted by Trizol method and subject to Northern blot analysis.

A: quantification of Northern blot in B. Values are given relative to 100% Ler-0 EtOH

B: Nothern blot hybridized with AGP31 probe

Ler-0: wild type plants

coi1-1: jasmonic acid signaling mutant. F-box protein, encoding subunit of E3 ubiquitin ligase SCF complex. abi1-1, abi2-1,abi3-1 same as Figure 2.4

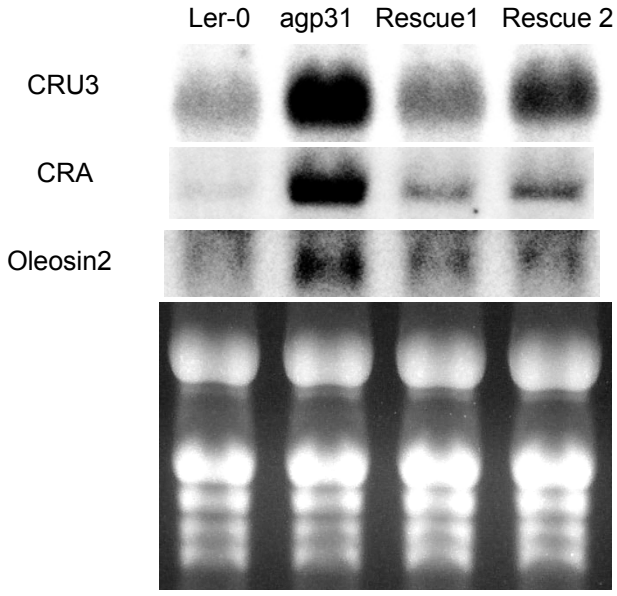


Figure 2.6 Seed storage protein expression

RNA was extracted from 7-day-old seedling growing in half MS agar plates and subjected to Northern blot analysis.

CRU3: cruciferin 3, CRA: cruciferin A, Oleosin 2

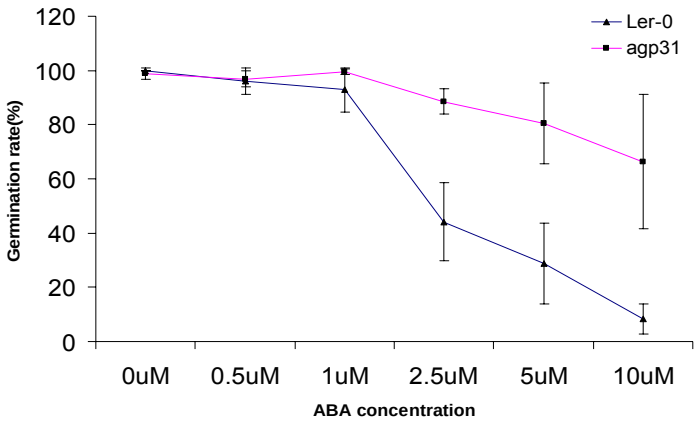


Figure 2.7 *agp31* mutant seed germination is resistant to ABA inhibition

Seeds from Ler-0 and AtAGP31 were sown on ½ MS 1% sucrose agar plates containing indicated concentration of ABA. The plates were placed at 4 °C for 2 days to break the dormancy. Then the seeds were germinated at 22 °C with continuous light. The germination rates were scored after 4-6 days. Values are plotted from the average of three independent batch seeds, error bars show one standard deviation.

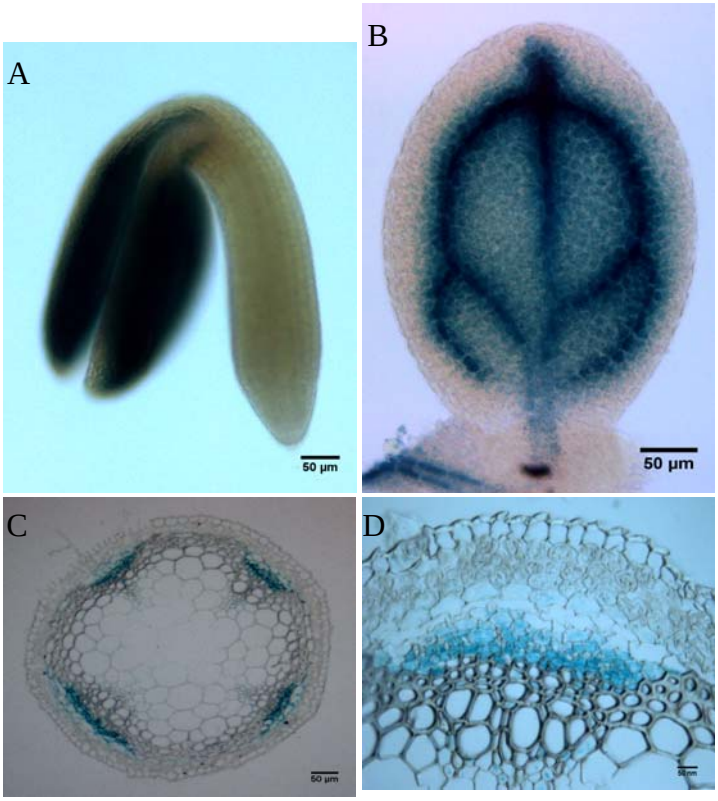


Figure 2.8 GUS staining to show AGP31 expression in seed embryo

AGP31 promoter::GUS construct was transformed into wild type plants. Embryo of about 15 days after pollination was dissected out from silique and stained with GUS substrate. A: embryo B: cotyledon of embryo Figure C,D: cross section of inflorescence stem C. cross section of inflorescence stem D. Close-up view of one vascular bundle of inflorescence shoot.

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